Perinatal motor function loss in human spina bifida aperta

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CHAPTER 1

General introduction

INTRODUCTION

Neural tube defects: spina bifida, anencephaly, and encephalocele

Neurulation disorders are congenital malformations characterized by defective fusion of the neural tube during the first weeks of pregnancy (3rd to 4th week of gestation).[1,2] The spectrum of neural tube defects consists of anencephaly, spina bifida and encephalocele. In anencephaly, the most severe neural tube defect, the cephalic end of the neural tube fails to close resulting in absent cranium and brain in the fetus. Anencephaly is not compatible with life.

In children with encephalocele, there is a smaller fusion defect of the cranium resulting in protrusion of brain tissue through an opening in the cranium in a saclike structure of meninges and skin. The treatment for encephalocele is operative by repositioning the brain tissue inside the cranium and removing the sac structure.

In children with spina bifida, the neural tube defect results in incomplete development of vertebrae. Spina bifida can be broadly divided in two forms: closed (occulta) and open (aperta) spina bifida (SBA). In spina bifida occulta, the spinal defect is completely covered by skin (no exposure of neural tissue). In SBA children, the incomplete fusion of the posterior vertebral arches is accompanied by a skin defect and a cerebrospinal fluid containing cele that protrudes through the bony defect. The two most common forms of spina bifida aperta are myelomeningocele (MMC) and meningocele. In children with MMC, the most common and most severe form of SBA, the protruding cele contains meninges and neural tissue including spinal cord and nerve roots. In children with meningocele, there is protrusion of meninges through the vertebral defect but no protrusion of spinal cord.

Pathogenesis of neural tube defects and the role of folic acid

Spina bifida concerns a heterogeneous patient group. The underlying cause is multifactorial, implicating that many factors may relate to the pathogenesis and/or severity of the defect. The main categories of risk involve hereditary, nutritional and environmental factors. This variety in contributional factors can be explained by the involvement of modifying genes, which can change the expression of the underlying defect. It has been shown that folic acid supplementation and dietary fortification can decrease the incidence of spina bifida. This is explained by several folate-dependent processes, such as DNA synthesis and the remethylation pathway of homocysteine to methionine.[3] Adequate folate levels are critical for cell division and when cell turnover is impeded during a critical period, it may result in a neural tube defect.

Results of a trial conducted by the Medical Research Council in 1991 showed that improving the periconceptional folate status in women would prevent 72% of all neural tube defects.[4] In the Netherlands, an official policy that advises women to take periconceptional folic acid supplementation (four weeks before conception until eight weeks after) was introduced by the government in 1993 with associated health education campaigns in 1995.[5] In 2005, 51% of the pregnant women in the Northern Netherlands used folic acid for the entire advised periconceptional period and 80% used folic acid in a part of the advised period (compared to 5% and 25%, respectively

in 1995).[5,6] As a result of the increasing folic acid awareness, the prevalence of neural tube defects declined significantly between 1992 and 2007 (Eurocat Europe data for the Northern Netherlands, update December 2009). However, this decline mainly concerned the incidence of anencephaly whereas the incidence of spina bifida was hardly affected.

Incidence of spina bifida in the Netherlands

In the Netherlands, there are about 185.000 life births a year (Centraal Bureau voor de Statistiek, update 16 July 2010). Approximately, one out of 1000 children is born with a neural tube defect. In the three Northern provinces (Groningen, Friesland, Drenthe), there are about 18.000 life births a year (Eurocat database, update 21 April 2010). Between 2002 and 2009, SBA was diagnosed in 73 fetuses in the Northern provinces of the Netherlands: 55% (n=40) of them were born alive, of which 16 deceased after birth; seven pregnancies ended in stillbirth and 36% (n=26) of the spina bifida pregnancies were terminated.[1] Introduction of the structural ultrasound examination in 2007 at the 20th week of pregnancy resulted in an increase of prenatally discovered cases of spina bifida up to 100% in 2008 (mean 66% between 1999-2008). In addition, there was an increased number of terminated spina bifida pregnancies before the 24th week gestational age. Between 2002 and 2007, 29% of the pregnancies were terminated compared to 48% between 2007 and 2009. All together, the reduction of live birth prevalence of neural tube defects is more likely to be caused by prenatal screening and pregnancy termination and not by primary prevention with folic acid supplementation.

Spina bifida aperta

This thesis concerns the children with SBA and MMC. The presence of a MMC is associated with motor-, sensory-, and autonomic function loss at, and caudal to the segmental level of the spinal defect. This neurological impairment is primarily due to the congenital malformation of the myelum, affecting neural innervation of the muscles, resulting in histological muscle impairment. In most SBA children, the MMC is accompanied by cerebral and spinal malformations including hydrocephalus, Chiari-2 malformation, diastematomyelia, and syringomyelia. These malformations could also impair motor functioning of the upper extremities, especially the fine motor skills.[7] Motor functioning in SBA children is thus related to both spinal and cerebral abnormalities. In this thesis, the terms '*cranial*' and '*caudal*' to the MMC refer to myotomes or dermatomes that have segmental innervations above (cranial) or below (caudal) the spinal segmental upper boundary of the MMC (as assessed by MRI).

Perinatal motor function loss in spina bifida aperta

Although SBA is associated with a neurological deficit caudal to the segmental level of the MMC,

1 Unpublished results of EUROCAT Registration 2002-2009 - with permission of dr. H.E.K. de Walle, epidemiologist Eurocat, department of Medical Genetics, University Medical Centre Groningen, The Netherlands.

fetal leg movements are still observable by ultrasound.[8-10] Most SBA fetuses show abnormal leg movements in utero[11] but they can even be of normal quality compared to healthy control fetuses.[8,9] However, leg movements by myotomes caudal to the MMC often tend to disappear within the first weeks after birth.[9,12-14] In SBA newborns, these (transiently present) leg movements may interfere with accurate prediction of motor function.[9,11,13]

The early perinatal disappearance of SBA leg movements might be explained by the 'second-hit hypothesis'.[15] The 'first-hit' concerns the damage associated with the congenital neurulation defect.[16,17] In addition to the congenital defect, secondary damage is initiated by exposure of the spinal cord to the intra-uterine environment.[18] This secondary damage is caused by neurotoxic influences of the amniotic fluid,[19,20] direct mechanical trauma[21] and delivery-related damage (such as spinal haemorrhages)[18,22] to the open spinal cord.

In order to preserve motor function in SBA, the effect of caesarean section was previously investigated in several studies. One study revealed that caesarean section (before labour onset) could preserve segmental neurological function caudal to the segmental level of the MMC,[23] whereas other studies showed no beneficial effect.[24-26]

Another innovative method for neuroprotection is represented by fetal coverage of the MMC. In SBA, fetal surgery aims at preventing secondary damage to the open spinal cord by prenatal coverage of the MMC.[18,20,21,27,28] Although fetal surgery might reduce damage by the 'second-hit' of damage, fetal surgery cannot 'cure' the congenital defect itself. This implies that the expected effect by fetal surgery concerns amelioration instead of complete reversal of the damage.

Muscle ultrasound

Previous research has shown that the muscle ultrasound technique can be applied as a non-invasive, diagnostic tool to assess muscle integrity in children with neuromuscular diseases.[29-35] In SBA, the quantitative parameter muscle ultrasound density (MUD) is based upon histologic muscle alterations that evolve after hampered neuromuscular innervation by the defect. These muscle alterations are histologically reflected by reduced muscle water content, muscle fibre atrophy, fat deposition[36] and fibrosis.[37] By an increased reflection of the muscle ultrasound beam, these muscle alterations will result in a higher muscle echogenicity (i.e. increased MUD).[29-34,38,39] By quantification of MUD, muscles of children with neuromuscular diseases can be discerned from normal healthy muscle tissue (with a low echogenicity).[34,40] Quantification of MUD has been applied in several ultrasound studies.[34-36,38,41-43] The MUD parameter in this thesis is determined according to the muscle ultrasound protocol described by Maurits et al.[34,38] In perspective of the above, we determined SBA leg-MUD in different myotomes as parameters for muscle integrity.

Aim of the thesis

The aim of this SBA thesis was to obtain insight in the perinatal pathogenesis (onset and progression) of leg muscle damage in relation with leg muscle function loss. For this approach we

used different diagnostic approaches, involving leg muscle ultrasound, post-mortem histological studies, radiological and neurological examinations.

Outline of the thesis

In **chapter 2**, we introduce a new fetal application of the well-known muscle ultrasound technique. We investigate whether fetal muscle ultrasound density parameters reflect muscle alterations caudal to the MMC. In **chapter 3**, we characterise muscular, vascular, and spinal pathology in SBA fetuses and its relationship with early neonatal motor function loss. In **chapter 4**, we investigate the pattern of leg-MUD alterations from birth until the first year of life. During this period, we would specifically expect that leg muscle alterations by the 'second-hit' of damage would come to existence. In **chapter 5**, we investigate the pattern of leg-MUD alterations from the first until the 18th year of life. During this period, we would expect that the impact by the 'second-hit' of damage upon histological muscle alterations would become stabilised. In **chapter 6**, we investigate whether visual dMUD assessment could provide a screening tool for estimation of leg muscle impairment by the MMC. If so, visual dMUD assessment could provide a global, fast and easily applicable neuromuscular screening tool. In **chapter 7**, we describe the comparative results between fetal endoscopic and postnatal MMC surgery. By age- and lesion matched comparison of leg-dMUD parameters, we obtained insight in the neuroprotective effects by both fetal and neonatal MMC closure strategies.

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CHAPTER 2

Muscle Ultrasound Density in Human Fetuses with Spina Bifida Aperta

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ABSTRACT

Background: In fetal spina bifida aperta (SBA), leg movements caudal to the meningomyelocele (MMC) are transiently present, but they disappear shortly after birth. Insight in the underlying mechanism could help to improve treatment strategies. In fetal SBA, the pathogenesis of neuromuscular damage prior to movement loss is still unknown. We reasoned that prenatal assessment of muscle ultrasound density (fetal-MUD) could help to reveal whether progressive neuromuscular damage is present in fetal SBA, or not.

Aim: To reveal whether prenatal neuromuscular damage is progressively present in SBA.

Patients/Methods: In SBA fetuses (n=6; 22-37 weeks gestational age), we assessed fetal-MUD in myotomes caudal to the MMC and compared measurements between myotomes cranial to the MMC and controls (n=11; 17-36 weeks gestational age). Furthermore, we intra-individually compared MUD and muscle histology between the pre- and postnatal period.

Results: Despite persistently present fetal leg movements caudal to the MMC, fetal-MUD was higher caudal to the MMC than in controls ($p<0.05$). Fetal-MUD caudal to the MMC did not increase with gestational age, whereas fetal-MUD in controls and cranial to the MMC increased with gestational age ($p<0.05$). In 5 of 6 patients assessed, comparison between pre- and postnatal MUD and/or muscle histology indicated consistent findings.

Conclusions: In fetal SBA, persistent leg movements concur with stable, non-progressively increased fetal-MUD. These data may implicate that early postnatal loss of leg movements is associated with the impact of additional neuromuscular damage after the prenatal period.

Abbreviations: SBA = spina bifida aperta; MMC = meningomyelocele; MUD = muscle ultrasound density

INTRODUCTION

In spina bifida aperta (SBA), defective neurulation is associated with a motor deficit caudal to the meningomyelocele (MMC).[1] Notwithstanding eventual postnatal motor function loss, perinatal leg movements are often still transiently present.[1-3] Insight in the underlying mechanism could help to improve treatment strategies. Several explanations for the disappearance of leg movements have been proposed. During embryogenesis, skeletal muscles develop from paraxial mesoderm at the dorsal part of the somite.[4,5] As a consequence of the neural tube defect, early mesodermal muscle development may become hampered. In a fetal SBA study, we showed that muscle histology is affected from the first trimester onwards.[1,6] In addition to the congenital defect, neural exposure by the open MMC could also progressively hamper neural function (by the neurotoxic content of amniotic fluid and by traumatic mechanical forces).[2,7] In sheep fetuses with surgically removed vertebral arches, Meuli et al. showed that spinal cord exposure to amniotic fluid causes progressive neurological damage.[8] In order to protect vulnerable neural connections at the MMC, these findings induced fetal therapy by coverage of the MMC.[9,10] Until now, actual proof for preservation of motor function by prenatal coverage of the MMC is still unconvincing.[10-12] This could be attributed to the prenatal surgical procedure itself (by sub-optimal timing and/or iatrogenic damage).[11,13,14] However, we have also shown that neural conduction through the MMC is still present in un-operated human SBA neonates during the first week of life.[15] From this perspective, it still remains unclear whether fetal neuromuscular damage in human congenital SBA is similarly progressive as in operated spinal sheep fetuses.

Postnatal human muscle maturation is characterized by a gradual process with a decrease in water and an increase in peptide content of the muscle.[16] Under physiological circumstances, this corresponds with an increase in postnatal muscle ultrasound density (MUD).[17] MUD values in children with neuromuscular disorders exceed those of normal, age-matched controls by additional fat and collagen deposition.[6,18,19] In this perspective, MUD may provide a non-invasive diagnostic tool for the assessment of neuromuscular damage.[17,19-24] To the best of our knowledge, MUD has never been applied as a diagnostic tool in fetuses before. In fetuses with SBA, we reasoned that assessment of MUD could provide insight in the onset and progression of neuromuscular damage prior to movement loss. We hypothesized that if MUD caudal to the MMC is increased in a non-progressive way (compared to normal controls), stable (non-progressive) dysfunction by the congenital neurulation defect is likely to be involved. However, if MUD caudal to the MMC would increase with gestational age, superimposed secondary neuromuscular damage could also be involved. In order to obtain insight in the onset and progression of neuromuscular damage in fetal SBA, we assessed fetal-MUD cranial and caudal to the MMC and compared outcomes with age-matched fetal control myotomes.

PATIENTS

The medical ethical committee of the University Medical Center Groningen, the Netherlands, approved the present study. After informed consent by the parents, fetal-MUD was assessed in six SBA (22-37 weeks gestational age; median 34 weeks) and 11 control fetuses (17-36 weeks gestational age; median 28 weeks). MMC was at thoracic (n=1), lumbar (n=1) or lumbar-sacral (n=4) level. All six SBA patients were delivered vaginally. Three of six fetuses were spontaneously delivered (patient 4, 5 and 6) and the other three fetuses were delivered after induction (patient 1, 2 and 3; by prostaglandine-E2 medication and/or additional cephalocentesis, respectively). The three fetuses that were delivered after induction, died during delivery. In these patients obduction and histological muscle assessment were performed. Patient 4 died within two weeks after birth due to severe illness (by the consequences of extensive hydrocephalus, microcephaly and Chiari II malformation). Parents gave no permission for obduction. In the other two surviving neonates (patient 5 and 6), pre- and early postnatal MUD could be assessed. Clinical data are summarized in Table I. In all fetal controls, neurological pathology was absent.

Table 1. Clinical data of included SBA patients

Case nr	Fetal US at GA	Partus at GA	Level MMC	Cerebral pathology	FM level	Postnatal data	AS 3' and 5'	PM level
1	22	22	L_5-S_1	HC,ChII	L_5-S_1	H	†	†
2	37	37	L_5-S_1	HC,ChII,DG,B,EC	L_5-S_1	H	†	†
3	36	41	Th_{12}-L_2	HC,ChII	L_5-S_1	H	†	†
4	35	40	L_4-S_4	HC,ChII,MC	L_5-S_1	-	-	L_1-L_2
5	33	38	L_4-L_5	HC,ChII	L_5-S_1	US	9/10	L_2-L_3
6	32	38	L_5-S_1	ChII	L_5-S_1	US	6/8	L_5-S_1

Legends: SBA = spina bifida aperta, nr = number, US = ultrasound assessment, GA = gestational age in weeks, MMC = meningomyelocele, FM level = lowest segmental level of fetal motor behaviour, L = lumbar, S = sacral, Th = thoracic, HC = hydrocephalus, ChII = Chiari II malformation, DG = dysgyration abnormality, B= bleedings, EC = encephalocele, MC = microcephaly, H = histological muscle assessment, - = no data, AS = Apgar score, † = perinatal death, PM level = lowest segmental level of postnatal motor behaviour

METHODS

In accordance with the previously described method of MUD assessment in children,[17,20] we assessed fetal-MUD. To exclude for alterations of the ultrasound signal by the maternal abdominal wall and fetal position, we expressed fetal-MUD as a ratio between muscle- to bone- density: [mean muscle pixel value] / [mean bone pixel value]. In order to obtain fetal control data, we determined the cross-sectional relationship between fetal-MUD and gestational age in 11 healthy fetuses (17-36 weeks gestational age), first. Consecutively, we cross-sectionally assessed and compared fetal-MUD between SBA and age-matched control fetuses (six age-matched pairs; 22-38 weeks gestational age; median 33.5 weeks). In SBA, MUD cranial to the MMC can be altered by cerebral pathology, whereas

MUD caudal to the MMC can be altered by both cerebral and spinal (i.e. the MMC) pathology. By comparison of MUD caudal to the MMC with MUD cranial to the MMC (in relation with healthy age-matched controls), the impact of the MMC upon MUD caudal to the MMC can be derived.

We assessed MUD in arm- (biceps or triceps (C5-C8)) and leg- (quadriceps (L2-L4), tibialis anterior (L4-L5), gluteus (L4-S1), hamstrings (L5-S2) and calf (gastrocnemius or soleus (L5-S1)) muscles and categorized outcomes according to segmental muscle innervation (i.e. either cranial or caudal to the MMC). Additionally, we assessed the occurrence and quality of leg movements caudal to the MMC (see reference[2] for description of the methods). The time interval between the last prenatal ultrasound recording and birth varied between 0 to 6 weeks.

In five of six SBA patients, we were able to obtain postnatal muscle parameters to serve as intra-individual controls. These postnatal muscle parameters consisted of neonatal-MUD assessment (in 2/2 surviving patients) and muscle histology (in 3/4 obducted patients). In the two surviving patients, postnatal MUD data were assessed within four days. The time interval between pre- and postnatal MUD assessment was 5 and 6 weeks. The ratio of MUD before and after birth (perinatal-MUD ratio) is expressed as: [neonatal-MUD] / [fetal-MUD]. Since it takes more than 1-2 weeks before MUD increases after acute neuromuscular injury, a perinatal-MUD ratio of approximately 1.0 would indicate that fetal-MUD outcomes are reproducible (by the postnatal technique) and non-progressive.

In the succumbed fetuses, myotomes cranial and caudal to the MMC were histologically assessed. Post mortem time before autopsy was 0-3 days. Muscles were stained by haematoxylin-eosin (H&E) and qualitatively classified as "discretely abnormal" (incidental muscle fiber hypertrophy), "moderately abnormal" (pronounced muscle fiber a- and hypertrophy), or "severely abnormal" (muscle atrophy and interspersed fat and collagen deposition (fibrosis)). ATP-ase staining was applied for assessment of muscle fiber type differentiation and type grouping. We mathematically compared fetal-MUD caudal to the MMC with fetal-MUD in age-matched control myotomes, according to the formula: ([fetal-MUD caudal to MMC] / [fetal-MUD control] x 100%). Furthermore, we associated outcomes with histological assessments.

Statistical analysis was performed by SPSS version 12.0.1 (SPSS, Chicago, IL). For correlations between gestational age and fetal-MUD, Kendall's tau was used. The Mann Whitney Test was applied to compare fetal-MUD in the 2nd and 3rd trimester in controls and between SBA and age-matched controls.

RESULTS

Prenatal muscular assessments

In all six SBA fetuses, leg movements caudal to the MMC were present. Movement quality was abnormal in 4 of 6 fetuses (hardly discernible). In SBA, fetal-MUD caudal to the MMC (calf muscle) was higher than in age-matched controls (medians 0.41 (range 0.36-0.45) and 0.30 (range 0.14-0.44) respectively; $p<0.05$), figure 1a. In 5 of 6 SBA patients, segmental innervation of quadriceps muscle

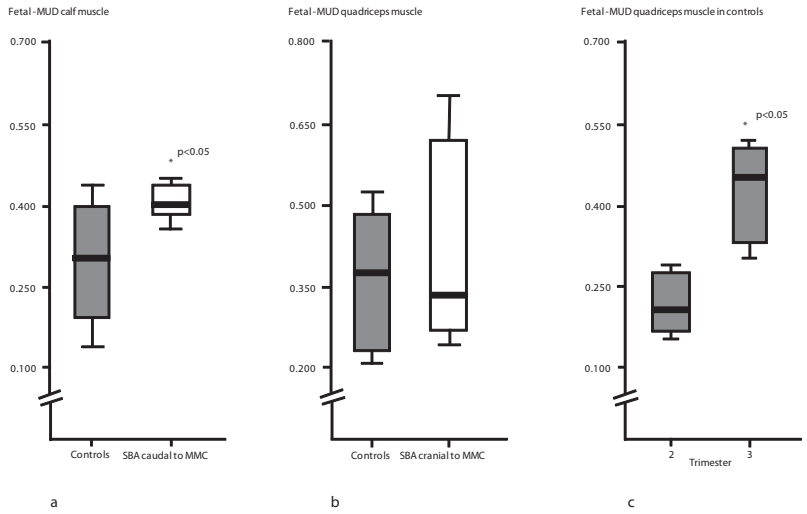

Figure 1 Fetal-MUD in SBA and control fetuses

(1a). Fetal-MUD in SBA caudal to the MMC compared with age-matched controls. The x-axis indicates fetal controls (left) and SBA fetuses (right). The y-axis indicates fetal-MUD of the calf muscle. Fetal-MUD reflects the mean pixel value of the muscle divided by the mean pixel value of the bone (pixel value range 0-255). Fetal-MUD in SBA (caudal to the MMC) is higher than in age-matched controls ($p<0.05$).

(1b). Fetal-MUD of quadriceps muscle (L2-L4) in SBA cranial to the MMC compared with age-matched controls. The x-axis indicates fetal controls (left) and SBA fetuses (right). The y-axis indicates fetal-MUD of the quadriceps muscle. Since comparison of fetal-MUD is performed between SBA fetuses with an innervation of the quadriceps muscle cranial to the MMC, only the 5 (of 6) SBA fetuses with a MMC at, or caudal to L4 are shown. Fetal-MUD in SBA cranial to the MMC does not significantly differ from fetal-MUD in age-matched controls.

(1c). Relationship between fetal-MUD and gestational age in controls. The x-axis indicates the trimester of pregnancy; the y-axis indicates fetal-MUD of the quadriceps muscle. Fetal-MUD is higher in the 3rd compared to the 2nd trimester of pregnancy ($p<0.05$).

MUD = muscle ultrasound density; SBA = spina bifida aperta; MMC = meningomyelocele; L = lumbar

was located cranial to the MMC. Comparing fetal-MUD in SBA cranial to the MMC (quadriceps muscle) with age-matched controls, indicated no significant differences (medians 0.34 (range 0.24-0.71) and 0.38 (range 0.21-0.52) respectively), figure 1b. In control fetuses, cross-sectional fetal-MUD of quadriceps muscle increased from the 2nd to 3rd trimester of pregnancy (medians 0.21 (range 0.15-0.29) and 0.46 (range 0.30-0.52) respectively; $p<0.01$), figure 1c. In SBA myotomes cranial to the MMC, fetal-MUD was also associated with gestational age ($n=5$; for quadriceps muscle: $r=0.50$; $p<0.05$). Fetal-MUD in SBA myotomes caudal to the MMC did not increase with gestational age ($n=6$; $r=0.26$; $p=0.13$).

Postnatal muscular assessments

Postnatal muscle parameters were obtained in 5 of the 6 SBA patients, consisting of neonatal-MUD or muscle histology (in 2/2 and 3/4 patients; respectively).

Intra-individually, perinatal-MUD ratio (i.e. [neonatal-MUD] / [fetal-MUD]) approximated 1.0 (1.0

- 1.3), figure 2a. Histological assessment varied between severely abnormal (patient 1: diffuse muscle fiber atrophy and interspersed fat and collagen deposition (fibrosis)), moderately abnormal (patient 2: pronounced muscle fiber a- and hypertrophy) or discretely abnormal (patient 3: normal muscle fibers with incidental fiber hypertrophy). In these patients, fetal-MUD caudal to the MMC in comparison with age-matched controls ([fetal-MUD caudal to the MMC] / [fetal-MUD control] x 100%) was 150% - 300% increased. The quantitative increase in fetal-MUD corresponded with the severity of histological alterations (i.e. patient 1, severely abnormal muscle alterations; patient 2, moderately abnormal muscle alterations; and, patient 3, discretely abnormal muscle alterations). ATP-ase staining did not indicate abnormal type grouping. Muscle histology did not indicate abnormalities in SBA muscles cranial to the MMC and fetal controls.

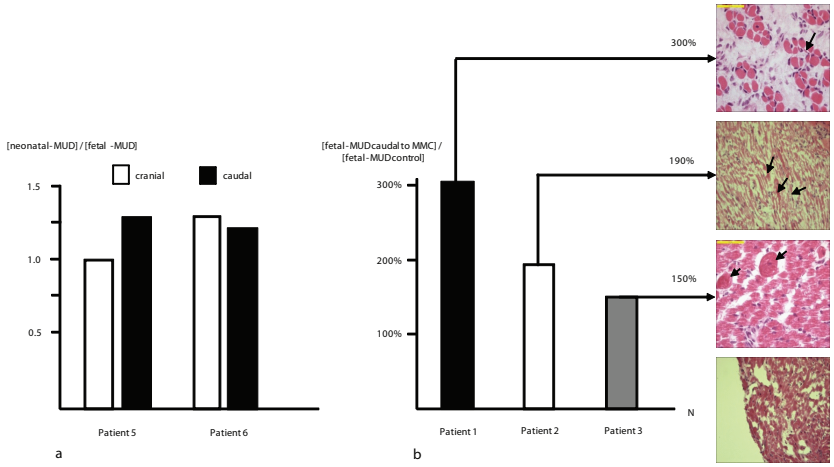

Figure 2 Longitudinal relationship between fetal and neonatal MUD and histology in SBA patients
(2a). Intra-individual fetal and neonatal MUD. The x-axis indicates neonate five and six that survived after birth. The y-axis indicates [neonatal-MUD] / [fetal-MUD]. Both cranial and caudal to the MMC, neonatal-MUD approximated fetal-MUD (indicated by: [neonatal-MUD] / [fetal-MUD] was 1.0-1.3). **(2b).** Relationship between fetal-MUD in SBA caudal to the MMC and histological assessment. The x-axis indicates all three neonates that died during delivery (patient 1, 2 and 3). The y-axis indicates the extent of fetal-MUD increase caudal to the MMC in relation to an age-matched control ([fetal-MUD caudal to MMC] / [fetal-MUD control] x 100%). On the right side, histology of corresponding muscles is shown (H&E staining). The extent of histological muscle damage appeared related with the proportionally increased fetal-MUD (compared to controls). Patient 1 (fetal-MUD increase of 300%) is associated with severely abnormal histology, patient 2 (fetal-MUD increase of 190%) with moderately abnormal histology and patient 3 (fetal-MUD increase of 150%) with discretely abnormal muscle histology. The associated histological assessments indicates interspersed collagen deposition and fibrosis (calf muscle; 22 weeks gestational age), pronounced fiber a- and hypertrophy (gluteal muscle; 37 weeks gestational age) and normal muscle fibers with an incidental fiber hypertrophy (paravertebral muscle; 41 weeks gestational age), respectively. At the bottom micrograph, a transverse section of a normal (N) paravertebral muscle is indicated. From this figure, it can be derived that the quantitative increase in fetal-MUD is related with the qualitative alteration in histology.
MUD = muscle ultrasound density; SBA = spina bifida aperta; MMC = meningomyelocele;
HE = haematoxylin-eosin

DISCUSSION

In human fetuses with SBA, we non-invasively assessed fetal-MUD to reveal the onset and progression of muscle damage caudal to the MMC. Our data indicate that fetal-MUD caudal to the MMC is stable and non-progressively increased compared with controls. These observations may implicate that the open defect at the MMC is more strongly associated with stable congenital neuromuscular alterations than with progressive fetal neuromuscular damage. To the best of our knowledge, fetal-MUD has never been assessed before. This technique may find a wider application for prenatal, non-invasive surveillance of other neuromuscular diseases.

In healthy children and adults, MUD has been shown to increase with gestational age.[17,20] Analogous to postnatal assessments, we also observed that fetal-MUD increases with gestational age (in myotomes cranial to the MMC and controls). Before the 20th week gestational age, healthy muscle fibers are still undifferentiated (type IIC). During the 20th- 30th week gestational age, muscle fibers mature from undifferentiated type IIc fibers into type II fibers, and, at term age into type I or type II fibers.[25] In healthy fetuses, muscle development involves a gradual process of a decreased water and increased peptide content,[16] corresponding with increased fetal-MUD. Thus, in fetal controls and SBA myotomes cranial to the MMC, the positive relationship between fetal-MUD and gestational age apparently reflects physiologic muscle maturation, whereas myotomes caudal to the MMC lack this relationship. It is well known that muscle damage is associated with a decline in water and increase in fat and collagen deposition in the muscle,[6,18] causing increased MUD compared with age-matched controls. Throughout gestation, fetal-MUD caudal to the MMC was non-progressively increased (compared with fetal-MUD cranial to the MMC and control myotomes). Present ultrasound findings seem confirmative of our previously published histological data indicating that fetal muscle alterations caudal to the MMC are non-progressively present.[1,6] Thus, these fetal data indicate that the open MMC is more likely to be associated with congenital, stable muscle alterations than with secondarily progressive neuromuscular damage.

We are aware that the present pilot data are obtained in a small number of patients. Despite this limitation, present results support the concept that fetal-MUD can provide a useful tool for non-invasive prenatal muscle assessment. In accordance with our previous observations, fetal leg movements persisted in all included SBA fetuses and disappeared shortly after birth.[1-3,6] These persistent fetal leg movements caudal to the MMC concurred with non-progressive prenatal muscle alterations. Shortly after birth, fetal leg movements caudal to the MMC disappear.[1-3] Since it takes more than 1-2 weeks before MUD increases after acute neuromuscular injury, it seems likely that the disappearance of postnatal leg movements is associated with acute neuromuscular damage after the prenatal period (for instance during delivery).[3,26]

In conclusion, in SBA, non-progressively increased fetal-MUD caudal to the MMC concurs with persistence of fetal leg movements. These data may implicate that early neonatal movement loss is caused by the impact of additional neuromuscular damage after the prenatal period.

Acknowledgements

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CHAPTER 3

Spinal hemorrhages are associated with early neonatal motor function loss in human spina bifida aperta

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ABSTRACT

Background: In spina bifida aperta (SBA), leg movements caudal to the meningomyelocele are present *in utero*, but they disappear shortly after birth. It is unclear whether leg movements disappear by impact of the neuro-developmental malformation or by superimposed traumatic damage. If superimposed traumatic damage is involved, targeted fetal intervention could improve motor outcome.

Aim: To characterize neuromuscular pathology in association with perinatal motor function loss in SBA.

Patients/methods: In fetal SBA (n=8; 16-40 wks GA), the median time interval between ultrasound registrations of fetal motor behavior and post-mortem histology was 1 week. Histology was assessed cranial, at and caudal to the meningomyelocele and compared with findings in fetal controls (n=4).

Results: Despite fetal movements caudal to the meningomyelocele (5/6), histology indicated muscle fiber alterations (6/6) that concurred with neuro-developmental and traumatic spinal defects [*Neuro-developmental defects:* spinal ependymal denudation (3/8), reduced amount of (caspase3-negative) lower motor neurons (LMNs; 8/8), aberrant spinal vascularization (8/8). *Traumatic defects:* gliosis (7/8), acute/fresh spinal hemorrhages near LMNs (8/8)].

Conclusion: In all delivered SBA patients, recent spinal hemorrhages were superimposed upon pre-existing defects. If early therapeutic strategies can prevent these acute spinal hemorrhages, motor outcome may improve.

INTRODUCTION

Spina bifida aperta (SBA) is characterized by defective fusion of the neural tube, resulting in exposure of the meningomyelocele (MMC) to the amniotic fluid. Leg movements by myotomes caudal to the meningomyelocele (MMC) are often still present *in utero*, but they disappear shortly after birth.[1-3] The early neonatal disappearance of leg movements has initiated fetal surgery to preserve leg movements.[4] However, although fetal closure of the MMC may reduce A. Chiari and hydrocephalus[5-7], results on potential preservation of motor behavior seem less convincing.[8-11] These disappointing results could be explained by the occurrence of lower motor neuron (LMN) damage in skin-covered spinal segments caudal to the MMC.[1,3] Thus far, however, the direct cause for early neonatal movement loss remains unclear. Theoretically, early neonatal motor function loss could reflect the impact by postnatal gravity upon the pre-existing neuro-developmental malformation.[2] Alternatively, early neonatal motor function loss could also reflect acquired traumatic damage superimposed upon the neuro-developmental malformation. In this respect, histological characterization of neuromuscular damage may help to identify the direct cause of movement loss. This information could provide relevant information for optimal timing and selection of treatment options. If the underlying neuro-developmental malformation is associated with movement loss, treatment would mainly focus on rehabilitation. If traumatic damage is superimposed upon the malformation, therapy could also aim at (fetal) prevention.[12] In SBA fetuses of various gestational ages, we reasoned that histological assessment of the spinal cord and associated myotomes could provide insight in the underlying cause for early neonatal movement loss.

PATIENTS

At the University Medical Center Groningen, we retrospectively investigated spinal and muscular histology in 8 SBA fetuses, which were autopsied between 1991 and 2006 (16-40 weeks GA). Parents of all included fetuses gave their informed consent. The medical ethical committee of our institute approved the investigation and analysis. The MMC was at cervical (n = 1), thoracic (n = 5) or lumbar (n = 2) level. After initiation of vaginal delivery, fetuses were born the same day. Vaginal delivery was associated with abruptio placentae (n = 1), misoprostol induction (n = 3) and cephalocentesis (n = 4). All four a-term, vaginally delivered patients were delivered in vertex position. Six fetuses died during delivery, two patients immediately thereafter. Clinical data are summarized in Table 1.

Spinal and muscular histology was also assessed in four fetal controls. The gestational age of the control fetuses (n = 4) varied between 22 and 41 (median 35) weeks. In these fetuses, delivery occurred spontaneously (n = 2), by caesarean section (n = 1) or by misoprostol induction (n = 1). Control fetuses had died from maternal keto-acidosis (diabetes), premature rupture of the amniotic membranes, umbilical cord strangulation and complicated twin pregnancy. Spinal or cerebral malformations were absent in all four control fetuses.

Table 1. Clinical information

Case	GA	MMC	Cause of death	Cerebral malf.	Orthopediac malf.	Other malf.
1	16	C-Th	misoprostolin-duction	cheilognato-palatoschisis holoprosencephaly	absent	pancreas annulare anal atresia
2	21	Th-L	misoprostolin-duction	ventriculomegaly, bleedings	absent	absent
3	22	L	misoprostolin-duction	ventriculomegaly Arnold Chiari	absent	triplication of the central canal
4	25	Th-L	solutio placentae	microcephaly ventriculomegaly Arnold Chiari	rocker bottom feet leg muscle contractures	Cardiomegaly abnormal lobulation of lungs
5	34	Th-L	induction, cephalocen-tesis	data incomplete	pes calcaneo-valg	lung hypoplasia, extrophia cloacae OEIS complex
6	37	L-S	induction, cephalocen-tesis	severe HC	absent	absent
7	40	Th-L	induction, cephalocen-tesis	absent vermis ventriculomegaly Arnold Chiari	pes calcaneovalg. fixed knee	palatoschisis, atrial septum defect
8	41	Th-L	induction, cephalocen-tesis	dysgyration, encephalocele Arnold Chiari	eversion feet	dilated ureters sacral agenesis

Legends: GA = gestational age, MMC= meningomyelocele, malf.= malformation, HC= hydrocephalus, C= cervical, Th= thoracic, L= lumbar, S= sacral, calcaneovalg.= calcaneovalgus, OEIS = omphalocele, exstrophy, imperforate anus, spinal defects.

METHODS

During the prenatal period, motor behavior was assessed by means of video-taped ultrasound recordings (n = 6). The time interval between video-recordings and delivery was 1 week (median, range 0-5 weeks). Two independent observers (D.A.S. and A.F.B.) assessed the quality of movements by Gestalt Perception.[13,14] According to previously described motor behavior characteristics in fetal SBA, movements were scored as: normal; poor repertoire (reduced variability); hardly discernible (i.e. minimal duration, small amplitude), and non-fluent (i.e. abrupt character).[2,3]

Histological data

Histology was assessed in: fused spinal segments cranial to the MMC (3/8), the cranial border of the MMC (i.e. ≤ 1 segment cranial to the MMC (7/8)), open spinal segments at the MMC (8/8), closed spinal segments caudal to the MMC (8/8) and subsequently also in corresponding myotomes (6/8). Post-mortem time before fixation ranged from 2 hours to 3 days after intra-uterine fetal death. The spinal cord was immersion fixed in a solution of 4% formalin in PBS (pH 7.4). To this solution some

NaCl was added to make the tissue float in order to overcome deformities of the tissue during the fixation period of 2 weeks. Transverse sections of spinal blocks were paraffin embedded and cut at 5 µm. Spinal abnormalities were subdivided into neuro-developmental defects or traumatic lesions. Histological staining of the spinal cord consisted of haematoxylin-eosin (H&E), cleaved caspase-3 (an apoptosis marker[15]), Nestin (for progenitor cells), GFAP (for gliosis) and CD68 (for macrophages and microglial cells). By H&E staining, the cellular quantity per motor neuron pool was estimated. If a spinal transverse section consisted of a motor neuron pool of less than 5 LMNs, the amount of LMNs was assessed as reduced. If a spinal transverse section consisted of a motor neuron pool of less than 3 LMNs, the amount of LMNs was assessed as severely reduced. Cleaved caspase-3 is a sensitive apoptosis marker that indicates the point at which the cell cannot return from the apoptosis cascade.[16] In this perspective, post-mortem investigation of spinal caspase-3 expression can reveal whether acute LMN cell death had been initiated recently days before, or not. We reasoned that if fetal leg movements are present despite caspase-3 positive LMNs, histological damage underlying neonatal motor function loss is initiated before birth. If fetal leg movements co-exist with caspase-3 negative LMNs, early neonatal motor function loss is initiated at a later time point (i.e. during or after delivery). For immuno-histochemistry, we applied: cleaved caspase-3 (1:1000, cell signaling technology, antigen retrieval (AR) of 1mM EDTA pH 9 in microwave at 700W for 8 min); nestin (1:100, Santa Cruz, AR using Tris/HCl at pH 9); and CD68 (1:100, kp1 clone, DAKO, AR using protease for 8 min). After the application of secondary and tertiary antibodies for 30 min, the slides were treated with diaminobenzidine and H_2O_2 for 10 min and counterstained with haematoxylin. Normal fetal spinal cord was used as control for the immunostaining procedures.

Muscle fiber type differentiation and type grouping (starting from the 24th week GA onwards[17]), were assessed by ATP-ase staining in all fetuses older than 24 weeks GA.

Statistical analysis

Wilcoxon signed-rank test was applied for comparison between the amount of LMNs cranial to the MMC with the amount of LMNs at or caudal to the MMC.

RESULTS

Fetal motor behavior

In 6 of 8 fetuses video-recordings of motor behavior were present with a median duration of one week prior to delivery. Muscle contractions caudal to the MMC were present in 5 of 6 fetuses (Table 2). Despite a small and short appearance, qualitative aspects were normal in 3 of 6 fetuses (case 2, 3 and 8). In two fetuses (case 1 and 6), the quality of leg movements caudal to the MMC was impaired (i.e. hardly discernible and abrupt (Table 2)). In the only fetus without movements caudal to the MMC (case 7), ankle and knee joints were immobile (Table 1) and spinal organization was severely abnormal (see next section).

Table 2. Association between fetal motor behaviour and spinal histology

Case	GA	MMC	Motor behaviour	Movement quality	LMN cran. B. MMC	LMN at / caud. MMC
1	16	C-Th	L5-S1	abrupt	N	R
2	21	Th-L	L5-S1	normal	N	SR, aberrant
3	22	L	L5-S1	normal	R; dystrophic	SR
4	25	Th-L	--	--	N	R; HE
5	34	Th-L	--	--	N	R
6	37	L-S	L5-S1	HD	N	R, aberrant
7	40	Th-L	absent	absent	--	absent
8	41	Th-L	L5-S1	normal	N	R

Legends: GA = gestational age, MMC= meningomyelocele, LMN= lower motor neuron, cran.B.= cranial border, caud.= caudal, C= cervical, Th= thoracic, L= lumbar, S=sacral, HD=hardly discernible, = no data; N = normal quantity (>5 LMNs/side/section); R = reduced quantity (3-5 LMNs/side/section); SR = severely reduced (< 3 LMNs/side/section); HE = hyper-eosinophilic

Spinal histology

Histology of the spinal cord was investigated and compared between: fused segments cranial to the MMC; the cranial border of the MMC; open segments at the MMC and fused segments caudal to the MMC (in 3/8, 7/8, 8/8 and 8/8 fetuses, respectively). In two fetuses, re-epithelisation of the neurulation defect suggested a smaller morphological size of the MMC than actually present. In all SBA fetuses, spinal integrity was better preserved cranial than caudal to the MMC (Fig. 1). At and caudal to the MMC, neuro-developmental defects consisted of: (severely) reduced number of LMNs (caspase3-negative, 8/8), abnormal localization of LMNs (2/8), aberrant spinal blood vessels (8/8, Figs. 1 and 2; see also next section), abnormal transitions from epithelium to connective tissue (2/8) and abnormalities of the central canal (6/8, Fig. 2), such as: abnormal shape (2/8), localization (1/8) and di-or triplication (3/8). In three fetuses, the ependymal cell lining between the central canal and underlying neuropil was partly absent, called "ependymal denudation"[18], Fig. 2. In two fetuses, immuno-histochemistry staining (nestin, CD68 and GFAP) showed that ependymal denudation concurred with local loss of underlying neuropil (containing neural progenitor cells; Figs. 2 and 3), invasion of macrophages and subsequent astrogliosis. In all fetal histological assessments, LMNs were caspase-3 negative. Despite caspase-3 negative staining, the quantity of LMNs caudal to the MMC was ≥ 50% less than at the cranial border and/or cranial to the MMC ((8/8); $p<0.05$; Table 2). Acquired, traumatic spinal damage at and caudal to the MMC was indicated by astrogliosis (7/8; Fig. 3) and by old- (2/8) or recent- (8/8) spinal hemorrhages (Figs. 2 and 3).

Vascular histology

Vascularization was normal in fetal controls ($n = 4$) and in SBA fetuses at cranial distance from the MMC ($n = 3$). However, spinal segments at the cranial border, segments at the MMC and segments

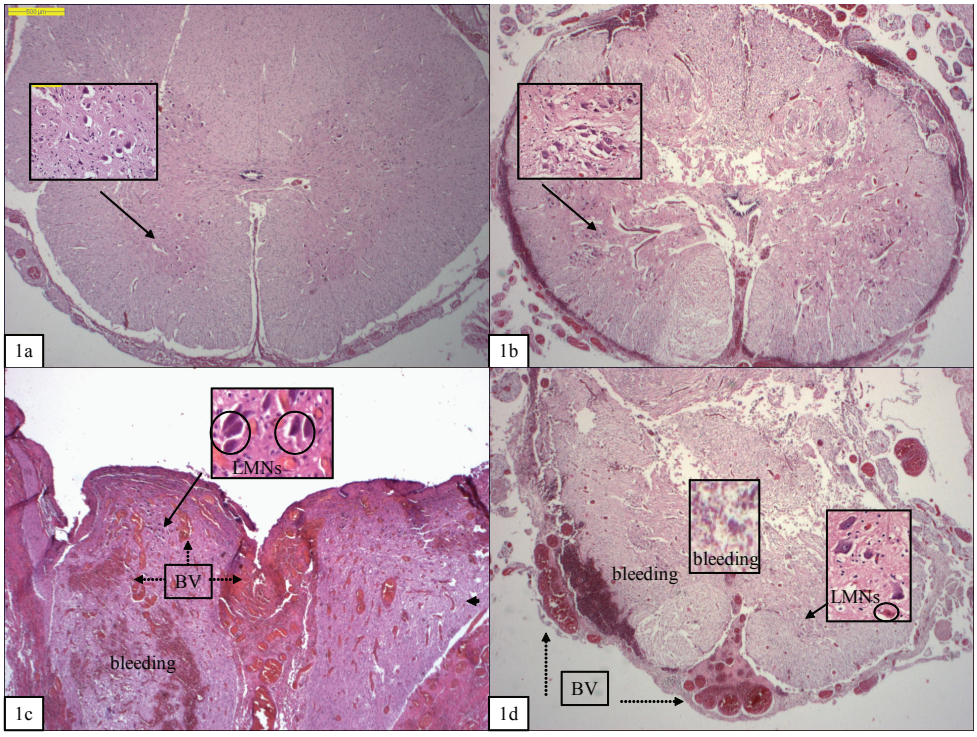

Figure 1. Cross-sectional images of the spinal cord.
1a: Cross-sectional image of the spinal cord in a control fetus 37 weeks GA. In the box, normal pools of LMNs are indicated.
1b: Cross-sectional image of the spinal cord cranial to the MMC in a SBA fetus of 25 weeks GA. In the box, normal pools of LMNs are indicated.
1c: Cross-sectional image of the spinal cord at the MMC in a SBA fetus of 37 weeks GA Aberrant blood vessels (indicated by dotted arrows) are present near ectopically located LMNs. An intramedullary hemorrhage is separately indicated.
1d: Cross-sectional image of the spinal cord caudal to the MMC in a SBA fetus of 25 weeks GA (identical fetus as in 1b). A small pool of LMNs with a hyper-eosinophilic LMN (encircled) is indicated. Spinal hemorrhages occur in the surroundings of LMNs. Dotted arrows indicate aberrant blood vessels (BV).
BV= abundant and aberrant blood vessels; H&E staining; augmentation: 10x and 20x.

caudal to the MMC consisted of superfluous, aberrant blood vessels (Figs. 1 and 2). Aberrant spinal blood vessels concurred with the appearance of fresh erythrocytes (i.e. recent hemorrhages) near caspase3-negative LMNs (8/8 fetuses; Figs. 1 and 2). These spinal hemorrhages were observed at closed spinal segments (at the cranial border of the MMC and caudal to the MMC) and at open spinal segments of the MMC (Figs. 1 and 2). In addition to recent hemorrhages, macrophages containing iron pigment (Perls stain positive) were observed (2/8 fetuses; indicative for hemorrhages of at least a few days old).

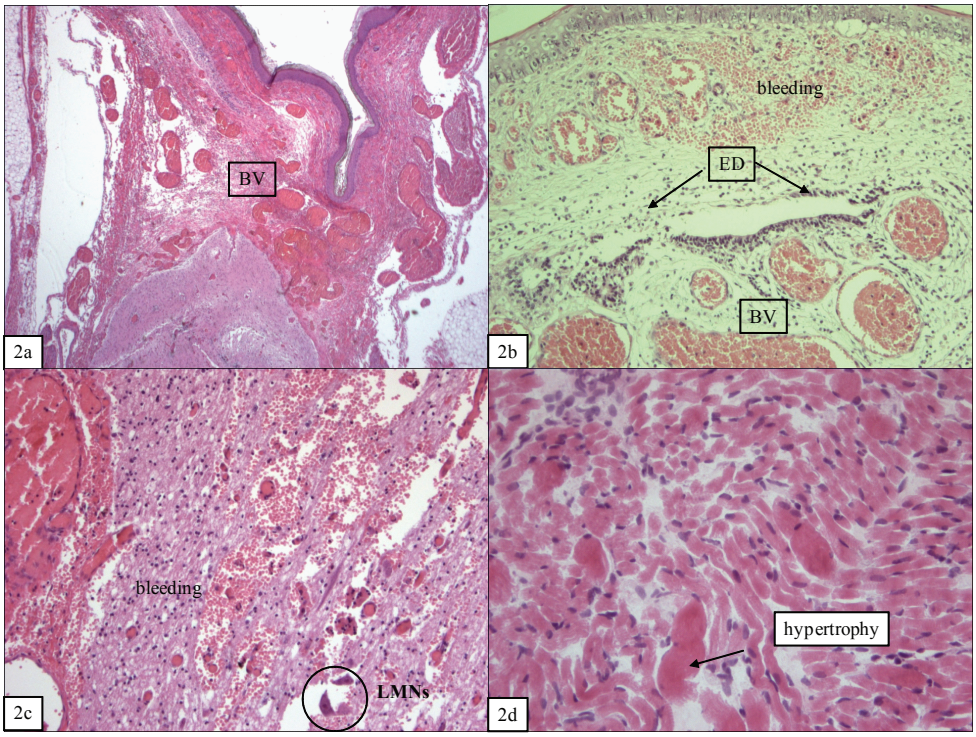

Figure 2. Spinal cross-sectional images in fetal SBA
2a: *Area medullovasculosa* caudal to the MMC in a SBA fetus of 37 weeks GA. Aberrant blood vessels are indicated.
2b: Abnormal shape of the central canal caudal to the MMC in a SBA fetus of 40 weeks GA. At the dorsal side of the central canal, ependymal denudation and a large, recent hemorrhage is indicated. At the ventral side of the canal, aberrant blood vessels are present.
2c: Cross-sectional image of the spinal cord caudal to the MMC in a SBA fetus of 37 weeks GA. A recent intramedullary hemorrhage adjacent to LMNs is indicated.
2d: Paravertebral muscle caudal to the MMC in a SBA fetus of 25 weeks GA. Hypertrophic muscle cells are indicated.
BV= abundant and aberrant blood vessels; ED= ependymal denudation; H&E staining; augmentation: 10x and 20x

Muscle histology

In six fetuses, muscle biopsies at myotomes cranial and caudal to the MMC were performed. In all six fetuses (22-40 weeks GA), muscle fibers in myotomes cranial to the MMC were normal, whereas muscle fibers in myotomes caudal to the MMC appeared a- and/or hypertrophic (Fig. 2). ATP-ase staining did not indicate type grouping in fetuses older than 24 weeks GA (5/5).

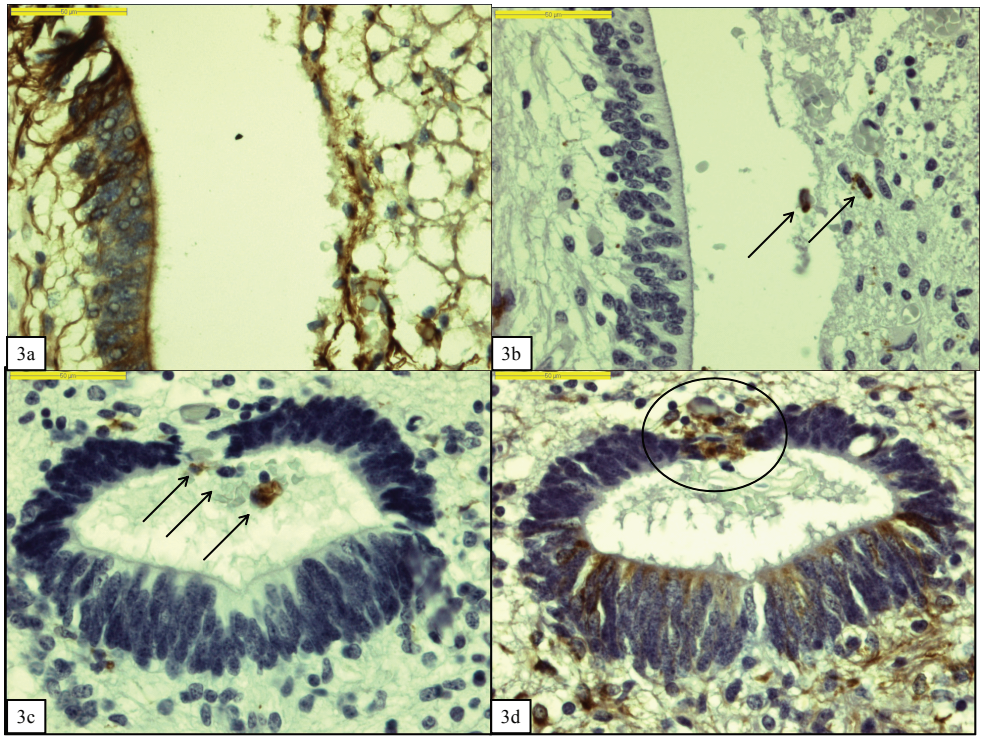

Figure 3.
3a: Nestin staining of a longitudinal section through the central canal in a SBA fetus of 40 weeks GA. At the right side of the central canal, the ependymal lining with underlying neuropil (containing progenitor cells) is completely lost along the length of the section.
3b: CD68 staining of a longitudinal section through the central canal in a SBA fetus of 40 weeks GA (same fetus as figure 3a). Macrophage invasion in the denuded area is indicated (arrows).
3c: CD68 staining of a transverse section through the central canal in a SBA fetus of 16 weeks GA. In the area with ependymal denudation, there is loss of progenitor cells and influx of macrophages (arrows).
3d: GFAP staining of a transverse section through the central canal in a SBA fetus of 16 weeks GA (same fetus as figure 3c). At the denudated area, astrogliosis is observed (encircled area).

DISCUSSION

In SBA, we characterized neuromuscular pathology in association with early neonatal motor function loss. Despite fetal neuro-developmental and (old) traumatic lesions, fetal leg movements caudal to the MMC persisted. Superimposed upon these non-progressive fetal defects, we observed delivery-related spinal hemorrhages that precede early neonatal movement loss.

At the 24th day of gestation, primary neurulation (i.e. closure of the neural tube) is a complex process occurring at multiple fusion sites of the neural tube.[19,20] In human fetuses, primary neurulation occurs in caudal direction between the cervical and caudal fusion point.[21] After completion of primary neurulation, mesenchymal cells divide and migrate into the tail bud (i.e. secondary neurulation at the conus area).[22,23] After primary and secondary neurulation at different areas[24,25], mesenchymal induction (formation of blood vessels and muscles) takes place.

In accordance with unidirectional primary neurulation of the spinal cord, spinal organization and vascularization cranial to the MMC was essentially normal. These normal histological findings were contrasted by pathological findings observed in spinal segments at, and caudal to the MMC. Spinal pathology caudal to the MMC involved: reduced quantity of LMNs, aberrant spinal blood vessels and occurrence of ependymal denudation.

In contrast to our findings, a report on two SB fetuses with only "minor" histological abnormalities caudal to the MMC has previously been published (9 and 15 weeks GA; crown-rump lengths 4 and 11 cm, respectively).[26] However, in the latter study, the open MMC was used as a reference and observations in closed segments cranial to the MMC were lacking. Furthermore, assessments of vascular condition, LMN quantity, ependymal integrity and underlying neuropil were absent. Until now, ependymal denudation was described exclusively at cerebral level[18], but, to the best of our knowledge, never at spinal level. After induction of the floor plate by the notochord (at 4th week GA), ependymal differentiation occurs in a fixed temporal and spatial pattern.[27] The process is mediated by the organizer gene *Sonic Hedgehog*[28], which is also involved in neural tube closure.[29] These fetal ependymal cells secrete important molecules that are involved in neural proliferation and migration.[27] In addition to protein secretion, ependymal cells also function as a barrier between CSF and the underlying neuropil. When ependymal cells are lost, functional restoration is impossible and the underlying neuropil can migrate into CSF.[18] At cerebral level, ependymal denudation is followed by an invasion of macrophages into the denuded areas. This macrophage invasion will subsequently result in gliosis.[18] Accordingly, we also observed ependymal denudation at spinal level. In the spinal cord, ependymal denudation was also followed by: loss of underlying neuropil, invasion of macrophages and subsequent gliosis at the denudated area. In this respect, human ependymal denudation shows striking similarities with that in mutant Hyh mice.[30-32] In Hyh mice, ependymal denudation has been ascribed to a primary failure in the formation of cell junctions.[33] In analogy with these data, it is tempting to speculate that ependymal denudation in human SBA rather reflects a primary rather than secondary pathogenesis. In human fetal SBA, this would implicate that spinal damage is not only associated with exposure of the neural plate to toxic amniotic fluid[34], but also with pathological neuro-developmental processes in well covered spinal segments caudal to the MMC.

At all gestational ages, the above described spinal abnormalities corresponded with muscle fiber abnormalities (a- and/or hypertrophy) in myotomes caudal to the MMC. Nevertheless, fetal movements caudal to the MMC had still been present and recorded on videotape. The early neonatal disappearance of these movements is preceded in time by fresh (i.e. normal erythrocytes), delivery-related spinal hemorrhages near LMNs (that were still caspase-3 negative at birth). In rat models, the time window for neural apoptosis and caspase-3 activation has been studied. In rat brain, cleaved caspase-3 becomes increasingly positive between 6 and 24 h after a traumatic or hypoxic-ischemic insult.[35] In spinal cord, focal hemorrhage is associated with apoptotic motor neurons in chicken embryos[36] and rat[37]. In human, elevation of programmed cell death markers has been indicated in

spinal motor fore horn diseases.[38] Accordingly, we have observed caspase-3 positive LMNs in a SBA neonate (with spinal hemorrhages) that had died two days after birth.[1] However, in the present study, fetal LMNs were still caspase-3 negative (8/8). From these studies, it seems apprehensive that LMNs may become caspase-3 positive only hours after delivery-related spinal hemorrhage. Delivery-related spinal hemorrhages were present at, and caudal to the MMC and were independent of presence or absence of asphyxia and prostaglandin induction (prostaglandins could even attenuate hemorrhages[39]). Despite traumatic influences during delivery in both control and SBA fetuses, spinal hemorrhages were absent in control fetuses (4/4) and were present in SBA fetuses (8/8). However, it is important to stress that included SBA fetuses did not represent an ad random, (relatively) favorable SBA study cohort which undergoes caesarean section (by a large, low transverse uterine incision[40]). In fact, all SBA fetuses were delivered vaginally to prevent maternal morbidity. In this perspective, we cannot exclude that traumatic influence (such as for instance cephalocentesis and protrusion of the amniotic sac[41-44]) had a negative impact on the MMC and its contents. However, spinal hemorrhages are not entirely explainable by a specific traumatic type of delivery. Firstly, hemorrhages occurred after both induction and spontaneous vaginal delivery (unpublished observation in a succumbed SBA neonate). Secondly, spinal hemorrhages were not only confined to the open location at the MMC itself, but were also observed in well covered segments caudal to the MMC. In accordance with our findings at the MMC, Meuli et al. also reported fresh hemorrhages at he MMC (i.e. aborted fetuses, 19-23 weeks GA; in absence of cephalocentesis).[45]

However, in the present study, we also observed considerably large bleedings caudal to the MMC (i.e. at unexposed, skin-covered segments (8/8)), suggesting that spinal hemorrhage is not only caused by direct exposure to amniotic fluid. If delivery trauma would be the solitary cause for spinal hemorrhages, an equal distribution between hemorrhages cranial and caudal to the MMC would be expected. However, spinal hemorrhages cranial to the MMC were absent (only at the cranial border of the MMC) and invariably present caudal to the MMC. These spinal hemorrhages were located near aberrant blood vessels. All together, spinal hemorrhages appear associated with the location of aberrant blood vessels, whereas traumatic delivery may be one of the factors that provoke them.

In histological SBA literature, the area with redundant spinal blood vessels at the MMC is called the *area medullovasculosa*.[46] In addition to presence of abnormal blood vessels, functional blood supply (blood flow and sheer wall stress) is also reported as inferior in SBA patients (compared with spinal cord injury patients).[47] During delivery, venous stasis (reduced venous return); direct mechanical compression (at the *area medullovasculosa)* and reduced arterial blood supply (by uterine labor contractions) can all provoke acute spinal hemorrhages. In accordance with these histological data, Luthy et al. reported that caesarean section before the onset of labor contractions provides a better motor outcome than caesarean section or vaginal delivery after the onset of labor contractions.[48] Hopefully, future research (for example by application of caspase-8 and -9, or FAS and FAS-ligand staining) may allow further quantification of neural damage after these acute spinal

hemorrhages.

In conclusion, our data in human fetal SBA indicate that fetal movements caudal to the myelomeningocele concur with pre-existing spinal defects. During delivery, acute spinal hemorrhages are superimposed upon these defects. If innovative fetal therapies[12] could target superimposed delivery-related spinal hemorrhages, motor outcome would be expected to improve.

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CHAPTER 4

In Spina Bifida Aperta, Muscle Ultrasound Quantifies the 'Second-Hit of Damage'

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ABSTRACT

Purpose: In spina bifida aperta (SBA), the 'second-hit hypothesis' explains for the occurrence of delayed spinal neural damage superimposed upon the congenital myelomeningocele (MMC). This may result in additional loss of perinatal leg movements. Innovative fetal surgery may (partly) prevent this, but results are hard to quantify in small groups concerning individually different patients. We reasoned that muscle ultrasound density (MUD) parameters could help to quantify neuromuscular consequences by the 'second-hit' of damage. In the present study, we therefore we aimed to reveal the association between quantitative leg-MUD alterations and leg motor function loss during the evolving impact by the 'second-hit of damage'.

Methods: In the present study, we assessed and compared leg-MUD parameters in 16 postnatally operated SBA infants MMC L_5 and 13 healthy controls. We cross-sectionally assessed SBA MUD *caudal* and *cranial* to the MMC in three age groups (0, 6 and 12 months postnatal age), and calculated the impact by the MMC at L_5 level as: $dMUD_{(L5)} = [MUD_{calf\text{-}muscle/S1\text{-}2}] - [MUD_{quadriceps\text{-}muscle/L2\text{-}4}]$.

Results: At 0 months, SBA leg-dMUD outcomes were higher than control outcomes ($p<.05$). Cross-sectional outcomes revealed an additional post-neonatal increase in SBA leg-dMUD (6 and 12 months of age; $p<.05$), corresponding with leg muscle function loss ($p<.05$).

Conclusions: In postnatally operated SBA, post-neonatally increased leg-MUD parameters appear reflective of the 'second-hit' of damage upon leg muscle function loss. We conclude that SBA leg-MUD parameters can quantify the consequences by the 'second-hit' of damage, thereby providing an objective quantitative evaluation tool for innovative neuro-protective treatment strategies.

INTRODUCTION

In spina bifida aperta (SBA) leg movements can still be observed before birth, but disappear shortly thereafter.[1-3] This phenomenon has been associated with the 'second-hit of damage' involving delayed, secondary spinal neural damage (for instance by spinal bleedings, neuro-toxicity and mechanical trauma) superimposed upon the congenital neural tube defect.[1,4-11] Innovative fetal therapies may ameliorate motor function loss by the 'second-hit of damage'.[12-15] Although the randomized controlled, multi-center MOMS (*Management Of Myelomeningocele Study)* trial[12,14] has proven therapeutic gain, smaller European study groups may still warrant more explicit quantitative neuromuscular validation.[16] Especially regarding inter-individual differences in lesions, graduations in muscle weakness and different iatrogenic complications, transparent and objective quantitative neuromuscular evaluation tools are required.[1-3,17,18]

The muscle ultrasound technique may provide such a quantitative tool for neuromuscular assessment.[19-25] In a prenatal SBA study, we have shown that the quantitative parameter "muscle ultrasound density" (MUD) is associated with histological muscle impairment (i.e. by neuropathological reduction of muscle water, fibrosis, fat deposition and atrophy[26]). Considering the time interval between muscle denervation (by the second-hit of perinatal damage) and subsequent histological muscle alterations, leg-MUD may be expected to reveal a quantitative increase after the first neonatal weeks to months. In this perspective, we would hypothesize that SBA myotomes could both reveal a *congenitally* increased MUD (by the neurulation defect itself) and, in addition to that, also a *secondarily* increased MUD (by the 'second-hit' of damage). We reasoned that if *secondarily* increased quantitative MUD associates with leg muscle function loss, muscle ultrasound could provide an individual quantitative evaluation tool for the 'second-hit' of damage, independent of the followed SBA treatment strategy.

PATIENTS AND METHODS

The medical ethical committee of the University Medical Center Groningen approved the study. With informed parental consent, we retrospectively compared leg-MUD parameters between 16 SBA (MMC at L_5 (range L_4-S_1)) and 13 control infants. SBA infants were born at 38 (35-40) weeks and controls at 40 (38-41) weeks gestational age (medians (ranges)). In all SBA infants, the MMC was closed during the first postnatal week. All SBA infants, except one, revealed a Chiari-2 malformation. Delivery mode involved vaginal delivery or cesarean section (11 vs 12 and 5 vs 1) for SBA and control infants, respectively. Cesarean section was either performed electively (2 vs 0) or after the initiation of labor (related with failure of delivery progression (3 vs 1)), for SBA and control infants respectively. Clinical data are indicated in Table 1. All control infants were delivered after an uneventful pregnancy in absence of perinatal complications or neurological abnormalities.

Muscle ultrasound and neurological assessments

We cross-sectionally assessed and compared MUD parameters in a total of 16 SBA and 13 control

Table 1: Clinical data of included SBA infants

Infant nr	AS at 1'and 5'	Upper level MMC	Shunt-dependency	nr of shunt dysfunctions at 6/12 months	Other cerebral pathology	Other spinal pathology
1	x/10	L_4	+	2/2	CCA	-
2	7/9	L_4	†	†	H	syrinx
3	9/10	L_4	+	0/1	CCA, HT	syrinx
4	x/x	L_4	+	2/2	-	syrinx, TC
5	8/9	L_5	+	3/3	-	syrinx
6	9/10	L_5	+	2/2	CCA, H	syrinx
7	5/9	L_5	+	2/2	CCA, H	syrinx
8	8/10	L_5	+	0/2	CCA	TC
9	4/8	L_5	+	4/4	H	syrinx, TC
10	6/9	L_5	+	1/1	HT	-
11	7/9	L_5	+	2/3	HT	syrinx
12	9/10	S_1	+	4/4	CCA	-
13	9/10	S_1	+	-	-	TC
14	x/x	S_1	+	4/4	-	syrinx
15	9/10	S_1	-	-	-	TC
16	6/8	S_1	-	-	-	syrinx, TC

Legends: SBA = spina bifida aperta, nr = number, AS = Apgar score, ' = minutes, x = missing data, MMC = myelomeningocele, L = lumbar, S = sacral, + = present, † = perinatal decease due to cerebral/cardiopulmonary instability, - = absent, CCA = corpus callosum agenesis, H = haemorrhage, HT = heterotopies, TC = tethered cord.

infants. Outcomes were compared between three cross-sectional age groups at 0, 6 and 12 months of age [0-2 (n=11); 4-8 (n=11) and 11-19 (n=15), respectively] and were associated with leg muscle function. We subsequently assessed intra-individual SBA MUD parameters (*caudal* and *cranial* to the MMC) in comparison with control data. We determined segmental MMC levels by spinal MRI. By deliberate selective inclusion of MMC levels at L_5 (MMC range L_4-S_1) only, MUD *caudal* to the MMC is represented by MUD of calf muscle (innervated by S_{1-2}, i.e. *caudal* to the MMC_{L5}) and *cranial* to the MMC is represented by MUD of the quadriceps muscle (innervated by L_{2-4}, i.e. *cranial* to the MMC_{L5}), see also figure 1. The *intra-individual* impact by the MMC upon MUD (dMUD) can thus be calculated as: dMUD at MMC-L_5 = [$MUD_{calf\text{-}muscle/S1\text{-}2}$] - [$MUD_{quadriceps\text{-}muscle/L2\text{-}4}$], figure 1.

All muscle ultrasound recordings were performed with *General Electric Healthcare LOGIQ 9* ultrasound equipment (Jiangsu, China) using a 14-MHz linear probe under standardized conditions.[22,23] According to standardized reference points, we recorded transverse ultrasound

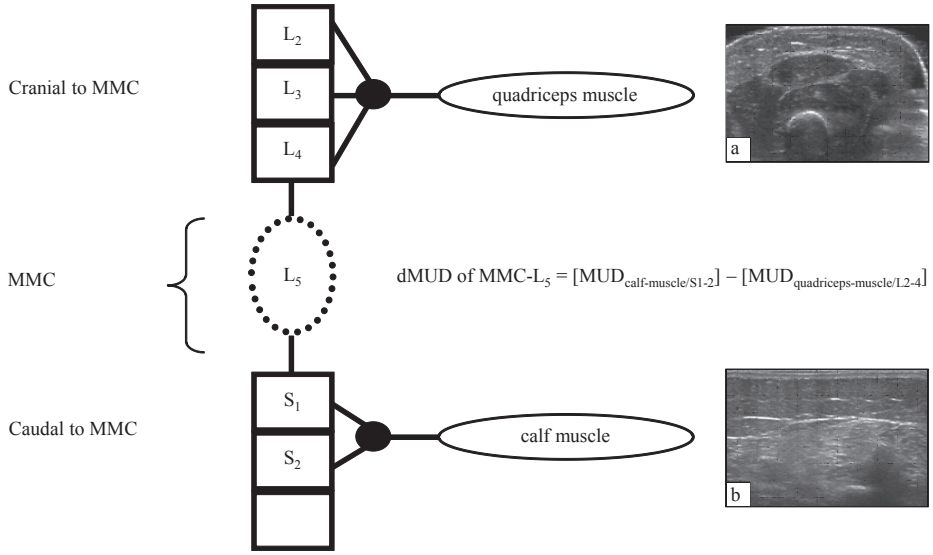

Figure 1: Schematic representation of myotome involvement cranial and caudal to the myelomeningocele.
In SBA-MMCL5, infants the quadriceps muscle (innervated by L2-4) is located cranial to the MMC and the calf muscle (innervated by S1-2) caudal to the MMC. To derive the effect by the MMC upon MUD caudal to the MMC, we calculated the intra-individual difference between MUD cadal and cranial to the MMC by: dMUD (MMCL5) = [MUDcalf-muscle/S1-2] -[MUDquadriceps-muscle/L2-4]. The images on the right side of the figure represent an example of corresponding muscle ultrasound images of the quadriceps (a) and of the calf muscle (b) in an infant with MMCL5.
SBA = spina bifida aperta; MMC = myelomeningocele; L = lumbar; S = sacral; MUD = muscle ultrasound density; dMUD = intra-individual difference in muscle ultrasound density.

images of the quadriceps muscle in supine (probe placed half-way between trochanter major and lateral knee joint cleft) and of the calf muscle in prone position (probe placed at position of maximum circumference). All muscles were recorded during muscle relaxation.[22,23] Since dMUD has to be substantial enough to be clinically relevant (i.e. visually detectable by the eye), we applied a cut-off point at 10 grey-values, representing the smallest intra-individual MUD difference that can be visually recognized by a clinical observer with a sensitivity exceeding 80%.
Muscle function caudal to the MMC_{L5} was represented by calf muscle function (innervated by S_{1-2}). An independent neurologist assessed motor function at the 0, 6 and 12 months postnatal time intervals.

Statistical analysis

We performed statistical analysis by PASW Statistics version 18.0 (SPSS, Chicago, IL). Since MUD values were not normally distributed (according to Q-Q plots and the Shapiro-Wilk test), we applied the non-parametric Mann-Whitney-U test for MUD comparison between SBA and controls. We compared intra-individual dMUD outcomes between SBA and controls by Chi-square-test. For the relation between MUD of the calf muscle and calf muscle function, we allocated all SBA infants to

two subgroups involving 1. functional (defined as: normal to mildly impaired) calf muscle function and 2. dysfunctional (defined as: absent to severely impaired) calf muscle function. We applied the Mann-Whitney-U test for the association between MUD parameters and calf muscle function. We estimated the time pattern of cross-sectional MUD alterations by one-way Anova. Statistical significance was set at α=.05.

RESULTS

The impact by the MMC (dMUD) at 0, 6 and 12 months postnatal age

At, and after the newborn period, dMUD was significantly higher in SBA than in corresponding control myotomes [0 months: 3 (-15-29) vs 0 (-5-10); 6 months: 22 (-6-40) vs 11 (-6-19) and 12 months: 18 (1-39) vs 8 (-6-13); medians (ranges); SBA and controls, respectively; dMUD = [$MUD_{calf\text{-}muscle/S1\text{-}2}$]-[$MUD_{quadriceps\text{-}muscle/\ L2\text{-}4}$] at cut-off point >10; *all p<.05*], figure 2.

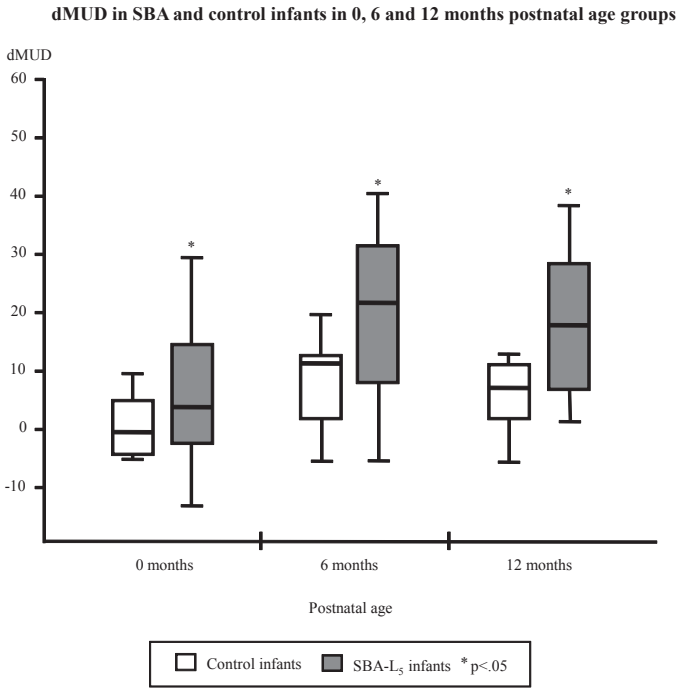

Figure 2: Intra-individual difference in muscle ultrasound density (dMUD) in spina bifida aperta and control infants.
The x-axis indicates 0, 6 and 12 months postnatal time intervals. The y-axis indicates the intra-individual difference in muscle ultrasound density between leg myotomes S1 and L2-4 (i.e. for MMCL5 caudal and cranial to the MMC, respectively). The intra-individual difference in muscle ultrasound density is calculated by: dMUD = [MUDcalf-muscle]-[MUDquadriceps-muscle]. At all ages studied, dMUD is higher in SBA than in corresponding control myotomes (p<.05).
dMUD = intra-individual difference in muscle ultrasound density; MMC = myelomeningocele; MUD = muscle ultrasound density; SBA = spina bifida aperta.

Cross-sectional relation between SBA MUD parameters and muscle function at 0, 6 and 12 months of age

After 0 months, SBA MUD *caudal* to the MMC increased [MUD calf muscle: t(23)=4.90; *p<.001*]. From 6 months onwards, SBA dMUD was higher in infants with dysfunctional than with functional calf muscles [6 months: 31 (16-40) vs 6 (-6-25) and 12 months: 27 (10-39) vs 5 (1-24); medians (ranges); absent vs present plantar flexion, respectively; *both p<.05*], figure 3a. From 6 months onwards, SBA MUD caudal to the MMC was higher in infants with dysfunctional than with functional calf muscles [6 months: 120 (94-133) vs 89 (82-104) and 12 months: 118 (72-145) vs 96 (70-113); medians (ranges); absent vs present plantar flexion, respectively; *both p<.05*], figure 3b.

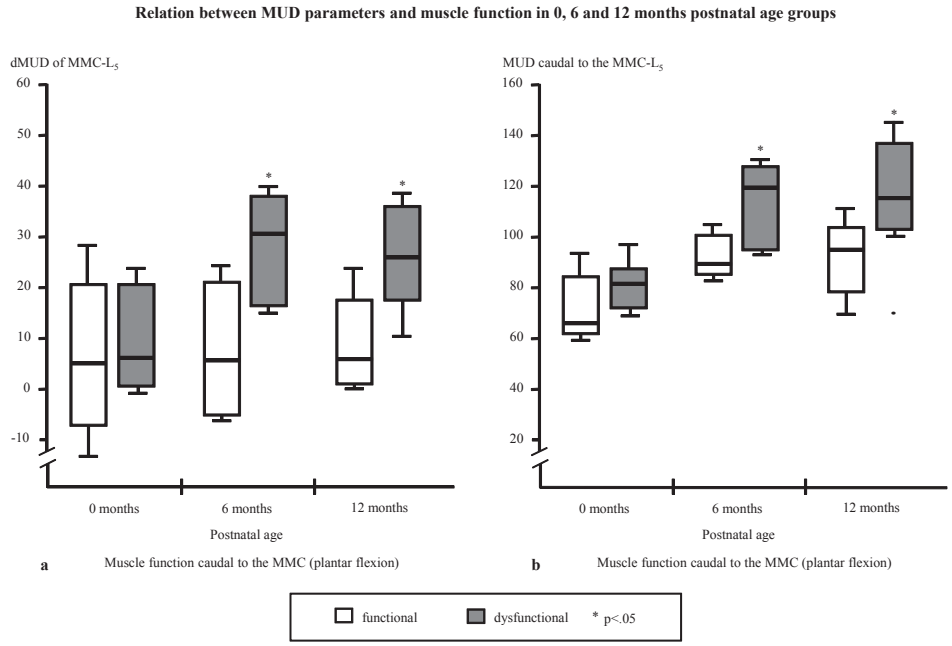

Figure 3: Relation between SBA MUD parameters and muscle function at 0, 6 and 12 months postnatal time intervals.
a: Relation between dMUD and plantar flexion of the foot.
The x-axis indicates postnatal time intervals. The y-axis indicates the intra-individual difference in muscle ultrasound density between leg myotomes caudal and cranial to the MMC (represented by: dMUD at MMCL5= [MUDcalf-muscle]-[MUDquadriceps-muscle]). The white and grey boxes indicate 'functional' and 'dysfunctional' plantar flexion of the foot. After the neonatal period, i.e. at 6 and 12 months postnatal age, dMUD is increased in the dysfunctional plantar flexion groups (p<.05).
b: Relation between MUD caudal to the MMC and plantar flexion of the foot.
The x-axis indicates postnatal age groups. The y-axis indicates MUD caudal to the MMC (i.e. MUD of the calf muscle). The white and grey boxes indicate 'functional' and 'dysfunctional' plantar flexion, respectively. After the neonatal period, i.e. at 6 and 12 months postnatal age, MUD caudal to the MMC is increased in the dysfunctional plantar flexion groups (p<.05).
dMUD = intra-individual difference in muscle ultrasound density; MUD = muscle ultrasound density; MMC = myelomeningocele; SBA = spina bifida aperta.

DISCUSSION

In SBA, the randomized MOMS trial has shown a significant neuro-protective treatment effect.[12,14] To evaluate the significance of the second-hit of damage for smaller European treatment groups, quantitatively objective neuromuscular parameters are required. Directly after birth, present results reveal higher dMUD outcomes in SBA than in controls, reflecting pre-existent 'congenital' leg muscle impairment by the neural tube defect, itself. After the neonatal period, we additionally observed a secondary increase in leg-MUD parameters, corresponding with postnatal leg muscle function loss. From these data, it may be deduced that secondarily increased quantitative SBA leg-MUD alterations are reflective of the impact by the second-hit of damage.[12,15,16,27] Interestingly, though, we observed some variation in SBA dMUD outcome parameters. This is likely to be explained by the large inter-individual heterogeneity of lesions (both cranial and caudal to the MMC), which induces variability. However, despite dMUD variation, results reached significance despite the relatively small SBA study group. Future assessment of individual *longitudinal* SBA dMUD outcomes may hopefully elucidate whether individual dMUD trajectories and MUD outcome parameters are applicable for individual longitudinal surveillance (to control for tethering), or not.

We recognize that there are several weaknesses to this study. Firstly, we studied a select SBA group with MMC_{L5} levels, only. We had deliberately chosen for this selective inclusion, so that bias by MUD assessment in heterogeneous muscle groups could be avoided. However, if we would compare dMUD in infants with different segmental MMC levels, we would hypothesize that similar results could be obtained, provided investigated myotomes are adapted to segmental levels cranial and caudal to the MMC. In perspective of the present, significant results in this well-selected, small group of MMC_{L5} lesions, we would therefore suggest that results should be regarded as indicative. Finally, we accepted a small age range between the 0, 6 and 12 months age groups. However, since MUD in healthy control children is age-independent,[28] we do not expect that this could have substantially influenced the presented results.

CONCLUSION

In SBA infants during the first year of life, postnatal quantitative SBA leg-MUD parameters can quantify the impact by the second-hit of damage upon leg muscle function. For relatively small European fetal treatment trials, these results may implicate that SBA MUD parameters can provide a useful quantitative evaluation tool.

Acknowledgments

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CHAPTER 5

Neurological Relevance of Pediatric Muscle Ultrasound in Spina Bifida Aperta

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Submitted

ABSTRACT

Objective: Spina bifida aperta (SBA) is associated with secondary perinatal spinal damage superimposed upon the 'congenital' neural tube defect ('second-hit damage'). This often induces asymmetrical secondary muscle impairment, which may be assessed by muscle ultrasound density (MUD). It is unclear whether potentially asymmetrical SBA leg-MUD alterations stabilize after the first year of life and remain indicatory for segmental neurologic function after the first year of life. If so, pediatric SBA leg-MUD outcomes after the first year of life could provide a non-invasive reference tool for segmental neurologic evaluation. In SBA children after the first year of life, we aimed to associate leg-MUD parameters with age and segmental neurologic function.

Study design: We included 23 SBA children [aged 4 (1-18) years, median (range); with a homogeneous myelomeningocele level at L_4-L_5 ($MMC_{L4\text{-}5}$)]. We determined leg-MUD outcomes for each leg and associated outcomes with: age, segmental leg function and outcomes in healthy controls [5 (1-17) years, median (range)].

Results: In SBA, leg-MUD parameters are: 1/ age-independent; 2/ higher than control values ($p<.05$); 3/ intra-individually asymmetrical ($p<.05$) and indicative for asymmetrical segmental leg function (sensory and motor dysfunction, both $p<.05$).

Conclusion: In SBA children after the first year of life, asymmetrical "stabilized" leg-MUD patterns can intra-individually provide age-independent reference values for the neuromuscular condition of each leg. This implicates that pediatric SBA-MUD data are relevant for intra-individual neuromuscular assessment.

INTRODUCTION

Perinatal spina bifida aperta (SBA) is associated with delayed spinal damage superimposed upon the 'congenital' neural tube defect ('second-hit' damage). By underlying 'secondary' perinatal spinal damage (involving delivery-related spinal hemorrhages,[1] mechanical and neurotoxic damage[2]), segmental neuromuscular outcome may differ between both legs.[3] Although spinal MRI may help to delineate the anatomic border of the myelomeningocele (MMC), it is an inaccurate indicator for asymmetrical leg muscle dysfunction.[4-6] However, such information is relevant for neurological follow-up and for determining functional consequences by tethering. Due to abnormal segmental innervation of myotomes caudal to the MMC, actual myopathic changes result in increased muscle ultrasound density.[7-10] In SBA under one year of age, we have shown that leg-MUD parameters can reflect episodes concerning the 'first' (fetal) and 'second' (perinatal) hit of damage in association with segmental outcomes.[7,11] After the first year of life, it is still unclear whether SBA leg-MUD changes stabilize after the second-hit of damage. If so, stabilized MUD parameters after the first year of life could provide a reference value for segmental surveillance of pediatric leg muscle integrity.

In the present SBA study, we reasoned that if intra-individually leg muscle function asymmetry is present, SBA leg-MUD outcomes would reveal the same pattern. In SBA children after the first year of life, we therefore aimed to assess leg-MUD parameters for each leg separately. We associated leg-MUD with: 1. age, 2. control MUD, and 3. segmental neurologic (motor and sensory) function of each leg. In SBA children after the first year of life, we reasoned that "stabilized" leg-MUD patterns could intra-individually provide a non-invasive reference tool for segmental neurologic evaluation.

PATIENTS

The medical ethical committee of the University Medical Center Groningen approved the study. With informed parental consent, we cross-sectionally included leg-MUD parameters of 23 SBA [age 4 (1-18) years; median (range)] with a homogeneous MMC level at L_4-L_5 (MMC_{L4-5}) and 16 healthy control children [age 5 (range 1-18) years; median (range)]. SBA children were born at 38 (32-40) weeks gestational age by vaginal delivery (n=12) or caesarean section (n=11). Caesarean section was either performed electively (n=7) or after failed delivery progression (n=4). Clinical data are shown in Table 1. In all SBA children, the MMC was surgically operated during the first postnatal week. All control children were delivered after an uneventful pregnancy in the absence of perinatal complications and/or neurological deficits.

METHODS

Muscle ultrasound and neurological assessments

In both SBA and control children, we correlated leg-MUD parameters with age. We applied spinal MRI to demarcate the upper MMC level, resulting in a homogeneous inclusion of MMC_{L4-5} lesions. As previously explained, the impact by the MMC upon leg muscles is derived from the formula: dMUD = [$MUD_{caudal\text{-}to\text{-}the\text{-}MMC}$] minus [$MUD_{cranial\text{-}to\text{-}the\text{-}MMC}$].[7,12] This implicates that dMUD in MMC_{L4-}

Table 1 Neurological data of included SBA patients with MMCL4/5

Patient number	Upper level MMC	Shunt-dependency	Other spinal pathology	Cerebral malformations
1	**L_4**	+	syrinx	Ch-2, CCH
2	L_4	+	-	Ch-2
3	L_4	+	-	Ch-2, CCH
4	L_4	+	syrinx	Ch-2
5	L_4	+	syrinx	Ch-2, CCH
6	L_4	+	syrinx	Ch-2, CCH, HT
7	L_4	+	TC	Ch-2, CCH
8	L_4	+	-	Ch-2
9	L_5	+	-	Ch-2
10	L_5	+	-	Ch-2, HT
11	L_5	+	syrinx	Ch-2, CCH, HT
12	L_5	-	-	Ch-2, CCH
13	L_5	+	syrinx	Ch-2, CCH
14	L_5	-	syrinx	Ch-2
15	L_5	+	syrinx	Ch-2, CCH
16	L_5	+	TC	Ch-2
17	L_5	+	syrinx	Ch-2, FA
18	L_5	+	-	Ch-2
19	L_5	+	-	Ch-2
20	L_5	-	-	Ch-2, CCH
21	L_5	+	-	Ch-2
22	L_5	+	-	Ch-2, CCH
23	L_5	+	DM	Ch-2, CCH

Legends: SBA = spina bifida aperta, L = lumbar, MMC = myelomeningocele, TC = tethered cord, DM = diastematomyelia, Ch-2 malformation = Chiari-2 malformation, CCH = corpus callosum hypoplasia, HT = heterotopies, FA = falx agenesis.

$_5$ children is represented by: [$MUD_{calf\text{-}muscle/S1\text{-}2}$] minus [$MUD_{quadriceps\text{-}muscle/L2\text{-}4}$]. Since dMUD has to be substantial enough to be clinically relevant (i.e. visually detectable difference in echo-density),[7] we applied a cut-off point (10 grey-values) representing the smallest dMUD value that can be visually discerned with a sensitivity over 80%.[9] In each SBA child, we assessed leg-MUD and dMUD per leg and we intra-individually compared results between both legs. The intra-individual SBA leg with the highest MUD outcomes was denominated as the most impaired leg, whereas the leg with smallest MUD outcomes was denominated as the least impaired leg. Subsequently, we also identified the intra-individual SBA leg with the most and with the least severe segmental neurological (motor and sensory) dysfunction. We subsequently tested whether asymmetrical leg MUD outcomes were associated with asymmetrical segmental neurological (motor and sensory) SBA outcomes.

Finally, we determined whether asymmetrical segmental leg function could also be attributed to an asymmetrical spinal impact by the MMC as represented by asymmetrical leg dMUD outcomes. Since quantitative dMUD parameters are dependent upon MMC levels, we only included MMC_{L4-5} lesions (i.e. dMUD of a $MMC_{cranial\text{-}to\text{-}L2\text{-}3}$ would be ameliorated by an increase in quadriceps muscle (L_2-L_4) damage, when dMUD is calculated by: [$MUD_{calf\text{-}muscle/S1\text{-}2}$] minus [$MUD_{quadriceps\text{-}muscle/L2\text{-}4}$]). Finally, we calculate the impact by the lesional level of combined MMC_{L4-5} lesions, by comparing outcomes between MMC_{L4} and MMC_{L5} lesions, separately.

Furthermore, we cross-sectionally compared SBA leg-MUD (of the most and least severely impaired leg) with leg-MUD outcomes in controls. Since MUD outcomes between the left and right leg were similar in 13 healthy control children [$MUD_{quadriceps\text{-}muscle}$ 76 (36-101) versus 76 (33-95) and $MUD_{calf\text{-}muscle}$ 83 (31-107) versus 85 (27-104); for left and right leg muscle respectively; both NS], we systematically compared SBA leg-MUD with right-sided leg-MUD control values.

Handling and storage of MUD data

All muscle ultrasound recordings were performed by either the *GE Healthcare LOGIQ 9* (fixed) or *GE Healthcare LOGIQ e* (portable) ultrasound machines. Both ultrasound machines are compatible systems (Jiangsu, China) owned by the University Medical Center Groningen and are calibrated by GE technicians. To allow comparison between MUD parameters obtained by both machines, we calculated a conversion factor by linear regression of LOGIQ *9* and *LOGIQ e* MUD outcomes.[12] The MUD conversion equation is: $MUD_{logiq\ 9} = 37.262 + 1.368 * MUD_{logiq\ e}$ (r^2=.74).

We performed muscle ultrasound registrations with standardized settings for muscle ultrasound gain, dynamic range, compression, and time-gain compensation parameters.[8,13] According to standardized reference points,[13] we recorded transverse ultrasound images of the quadriceps muscle (in supine position) and of the calf muscle (in prone position) during muscle relaxation. We assessed digital MUD according to a standardized method.[7,8,12-14]

Neurological assessment

We performed neurological assessments of segmental motor and sensory function under standardized conditions (i.e. in a quiet and alert behavioral state of the child) during the same day as the ultrasound assessments. Muscle function caudal to the MMC_{L5} was represented by calf muscle function (innervated by S_{1-2}). Muscle function cranial to the MMC_{L5} was represented by quadriceps muscle function (innervated by L_{2-4}). Muscle function was scored as present when muscle force could be delivered against resistance. Segmental sensory levels were indicated by the lowest dermatome at which a pinprick elicited an emotional response. Segmental sensory function caudal to the MMC_{L5} was represented by present sensory function at, or caudal to dermatome S_1. Segmental sensory function cranial to the MMC_{L5} was represented by present sensory function at, or cranial to dermatome L_4. We separately assessed motor and sensory levels for the left and right leg.

Statistical analysis

We performed statistical analysis by PASW version 18.0 (SPSS Inc., Chicago, IL, USA). Since MUD values were not normally distributed (according to Q-Q-plots and Shapiro-Wilk tests), we used non-parametric correlation (Kendall's tau) for the association of leg-MUD and dMUD with age. For statistical comparisons between SBA and controls and for the association between segmental neurological motor and sensory dysfunction and MUD, we applied non-parametric Mann-Whitney-U tests. We compared dMUD outcomes between the most and least severely impaired leg by the Wilcoxon Signed-Rank test (matched pairs). In the most severely impaired legs, we finally tested whether muscle dysfunction caudal to the MMC was associated with a higher dMUD by the Mann-Whitney-U test. Statistical significance was set at α=.05.

RESULTS

Leg-MUD versus age (1-18 years)

In both SBA and healthy control children, leg-MUD and dMUD outcomes were un-related with age [$MUD_{calf\text{-}muscle/S1\text{-}2}$: r=.11 and r=.01; $MUD_{quadriceps\text{-}muscle/L2\text{-}4}$: r=.17 and r=.07 and dMUD: r=-.17 and r=-.03; in SBA and controls respectively; *all NS*].

Intra-individual SBA MUD assessments in both legs of SBA children compared to controls

In SBA children, intra-individual leg-MUD and dMUD comparisons revealed a significant difference between both legs [MUD *caudal* to the MMC: 130 (74-170) vs 118 (69-160); MUD *cranial* to the MMC: 103 (68-146) vs 97 (67-127) and dMUD: 26 (-14-73) vs 13 (-24-56); medians (ranges), in the most and least impaired leg, respectively; *all p<.001*]. In both the most severely- and in the least severely impaired leg, SBA leg-MUD exceeded control outcomes (both *caudal* and *cranial* to the MMC; *p<.05*).*

*Comparative outcomes between the most severely impaired SBA-leg versus control outcomes (figure 1a-c), involved: $MUD_{calf\text{-}muscle/S1\text{-}2}$ (*caudal* to the MMC): 130 (74-170) vs 85 (69-100); $MUD_{quadriceps\text{-}muscle/L2\text{-}4}$ (*cranial* to the MMC): 103 (68-146) vs 74 (64-91); dMUD: 26 (-14-73) vs 7 (-4-22); medians (ranges); all *p<.001;* SBA and controls, respectively. Comparative outcomes between the least severely impaired SBA-leg versus control-leg, involved: $MUD_{calf\text{-}muscle/S1\text{-}2}$ (*caudal* to the MMC): 118 (69-160) vs 85 (69-100); $MUD_{quadriceps\text{-}muscle/L2\text{-}4}$ (*cranial* to the MMC): 97 (67-127) vs 74 (64-91); *both p=.010;* dMUD: 13 (-24-56) vs 7 (-4-22); *p=.060*; medians (ranges); in SBA and controls, respectively.

Cross-sectional SBA leg-MUD data are associated with segmental neurological function

The leg with most neurologic impairment (i.e. muscle (I) and sensory dysfunction (II)) revealed higher leg-MUD outcomes than the leg with least neurologic impairment, both I and II *p<.05*.#

#Specific quantitative data of the most severely impaired leg (figure 2a-d), involved: I. MUD in absent versus present leg muscle dysfunction: $MUD_{calf\text{-}muscle}$: 137 (74-170) vs 100 (83-126); *p=.018* and $MUD_{quadriceps\text{-}muscle}$: 108 (68-146) vs 89 (72-127); *p=.062*; medians (ranges); absent vs present calf and

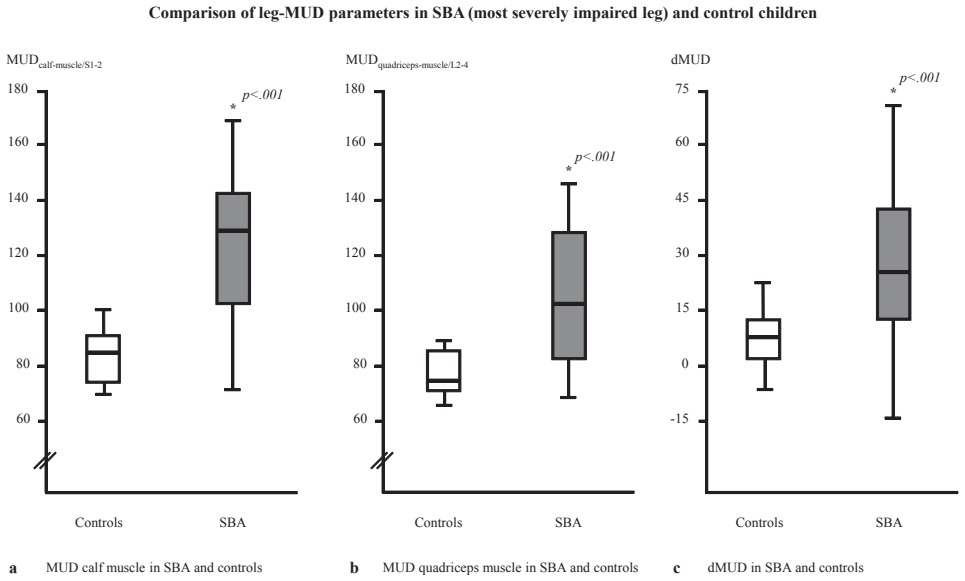

Figure 1: Comparison of leg-MUD parameters in SBA (most severely impaired leg) and control children.
a: MUDcalf-muscle/S1 (caudal to the MMC) in SBA and control children. In SBA, MUDcalf-muscle/S1 exceeds control values (p<.001).
b: MUDquadriceps-muscle/L2-4 (cranial to the MMC) in SBA and control children. In SBA, MUDquadriceps-muscle/L2-4 exceeds control values (p<.001).
c: dMUD in SBA and control children. dMUDL4/5 = [MUDcalf-muscle]-[MUDquadriceps-muscle]. dMUD is higher in SBA than in controls (p<.001).
MUD = muscle ultrasound density; SBA = spina bifida aperta; MMC = myelomeningocele; S = sacral; L = lumbar; dMUD = intra-individual difference in muscle ultrasound density.

quadriceps muscle function, respectively. II. MUD in absent versus present sensory perception in S_1 and L_4 segments, respectively: $MUD_{calf\text{-}muscle/S1}$: 135 (74-170) vs 97 (83-115); *p=.008* and $MUD_{quadriceps\text{-}muscle/L4}$: 122 (66-146) vs 79 (70-106); *p=.002*; medians (ranges); absent vs present sensory S_1 and L_4 perception, respectively.

Specific quantitative data of the least severely impaired leg involved: I. MUD in absent versus present leg muscle dysfunction: $MUD_{calf\text{-}muscle}$: 137 (94-170) vs 96 (69-126); *p=.001* and $MUD_{quadriceps\text{-}muscle}$: 122 (79-146) vs 76 (67-127); *p=.001*; medians (ranges); absent vs present calf and quadriceps muscle function, respectively. II. MUD in absent versus present sensory perception in S_1 and L_4 segments, respectively: $MUD_{calf\text{-}muscle/S1}$: 133 (74-162) vs 101 (83-139); *p=.037* and $MUD_{quadriceps\text{-}muscle/L4}$: 117 (68-146) vs 79 (70-106); *p=.005*; medians (ranges); absent vs present sensory S_1 and L_4 perception, respectively.

The impact of the MMC on dMUD and leg muscle function

Comparing SBA dMUD between MMC_{L4} and MMC_{L5} conditions, revealed significantly lower dMUD outcomes in MMC_{L4} than in MMC_{L5} lesions [18 (-14-30) vs 30 (6-73); medians (ranges); *p=.033*].

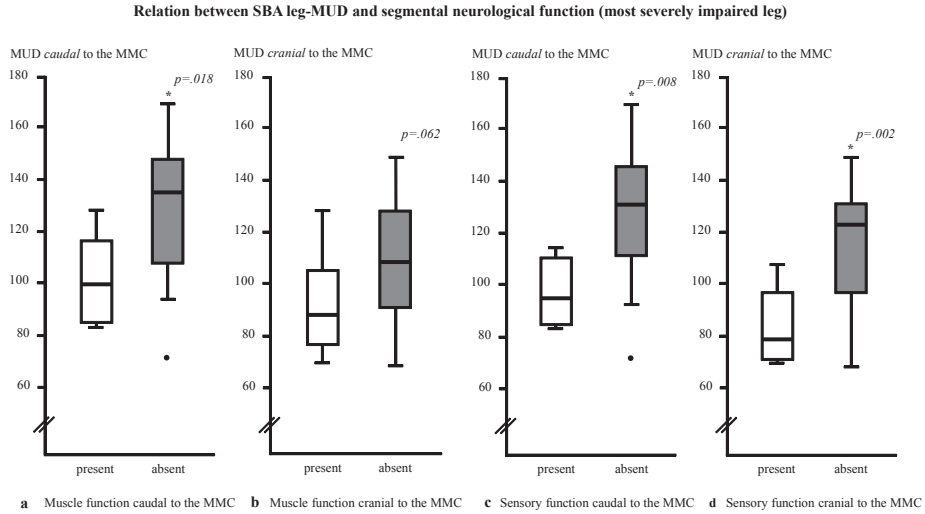

Figure 2: Relation between SBA leg-MUD and segmental neurological function (most severely impaired leg).
a: Relation between MUD caudal to the MMC and muscle function caudal to the MMC.
Present/absent: present/absent calf muscle function. MUD caudal to the MMC is represented by MUDcalf-muscle/S1 of calf muscle. MUD caudal to the MMC is higher in SBA children with absent than present calf muscle function (p=.018).
b: Relation between MUD cranial to the MMC and muscle function cranial to the MMC.
Present/absent: present/absent quadriceps muscle function. MUD cranial to the MMC is represented by MUD of quadriceps muscle. SBA MUD cranial to the MMC tended to be higher in children with absent than present quadriceps muscle function, but did not reach significance (p=.062).
c: Relation between MUD caudal to the MMC and sensory function caudal to the MMC.
Present/absent: present/absent sensory function in dermatome S1. MUD caudal to the MMC represented by MUD of calf muscle. SBA MUD caudal to the MMC is higher in children with absent than present sensory function in dermatome S1 (p=.008).
d: Relation between MUD cranial to the MMC and sensory function cranial to the MMC.
Present/absent: present/absent sensory function in dermatome L4. MUD cranial to the MMC represented by MUD of quadriceps muscle. SBA MUD cranial to the MMC is higher in children with absent than present sensory function in dermatome L4 (p=.002).
SBA = spina bifida aperta; MUD = muscle ultrasound density; MMC = myelomeningocele; S = sacral; L = lumbar; MUD caudal to the MMC is represented by MUDcalf-muscle; MUD cranial to the MMC is represented by MUDquadriceps-muscle.

Comparison of dMUD between the most and least severely impaired leg indicated higher dMUD outcomes in the most severely impaired leg [all MMC_{L4-5} lesions together: 26 (-14-73) vs 13 (-24-56); *p<.001*; subdivided for MMC_{L5} lesions: 30 (6-73) vs 23 (-16-56); *p=.001*; subdivided for MMC_{L4} lesions: 18 (-14-30) vs 5 (-24-29); *p=.012*; most and least impaired legs, respectively], figure 3a.

In $MMC_{L4,}$ statistical dMUD comparison between dysfunctional and functional calf muscles is impossible due to calf muscle involvement in both groups (intrinsic to calf muscle innervation L5-S1, i.e. caudal to MMC_{L4}). In MMC_{L5} lesions, dMUD was higher in the subgroup with dysfunctional than in the subgroup with functional calf muscles [for impaired legs: 47 (13-73) vs 18 (3-37); *p=.040*], figure 3b.

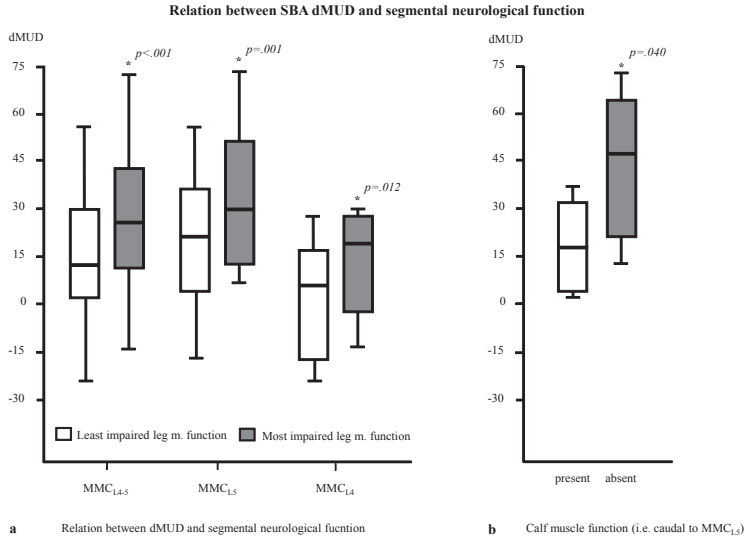

Figure 3: Relation between SBA dMUD and segmental neurological function.
a: Comparison of SBA dMUD outcomes between the most and least severely impaired leg.
SBA children, subdivided according to the MMC levels. MMCL4-5 refers to all included SBA children with MMCL4 and MMCL5 lesions. MMCL4 refers to SBA children with cranial demarcation of the MMC at L4. MMCL5 refers to SBA children with cranial demarcation of the MMC at L5. dMUD = [MUDcalf-muscle]-[MUDquadriceps-muscle]. Comparing legs with most and least impairment, revealed higher dMUD outcomes for most severely impaired legs (all p<.05).
b: MMCL5 - dMUD comparison between dysfunctional and functional calf muscles for most severely impaired legs.
dMUD = [MUDcalf-muscle]-[MUDquadriceps-muscle]). Asymmetrically impaired calf muscle dysfunction was associated with higher dMUD outcomes in most dysfunctional leg muscles (p=.040).
SBA = spina bifida aperta; dMUD = intra-individual difference in muscle ultrasound density; MMC = myelomeningocele; L = lumbar; MUD = muscle ultrasound density.

DISCUSSION

In SBA children after the first year of life, we aimed to associate leg-MUD parameters with age and asymmetrical segmental neurologic function. In SBA, intra-individually asymmetrical leg-MUD parameters are age- independent and associated with the segmental neurological function of each leg. In SBA children (1-18 years of life), these findings implicate the relevance of SBA-MUD data for intra-individual neuro-muscular assessment.

In SBA fetuses and infants until one year of age, we have previously shown that pre- and early postnatal leg-MUD alterations reflect the impact by 'congenital' and 'secondary' spinal damage upon leg muscle integrity.[11,12] After the first year of life, when the impact by the 'second-hit' of spinal damage is completed, we revealed a stabilization of MUD in association with asymmetrical segmental neurological (motor and sensory) function. Since cross-sectional pediatric MUD values are age independent (between 1 and 18 years of age), specific individually occurring traumatic

circumstances (such as tethering and/or secondary effects of aging or inactivity)[15-20] may thus become assessable by leg-MUD alterations. We will have to await our prospectively collected, longitudinal SBA MUD data to reveal whether such individual MUD alterations are clinically applicable for individual surveillance, or not.

Interestingly, both SBA MUD-*cranial* and -*caudal* to the MMC were increased in comparison with control values. This finding is explainable by the impact of cerebral damage upon myotomes both caudal and cranial to the MMC.[21-23] Since dMUD is composed of MUD *caudal* and *cranial* to the MMC,[24] this implicates that dMUD is also subject to the variability of lesions cranial to the MMC. This seems reflected by the presently observed relatively large range of dMUD outcomes. Another factor contributing to dMUD variability is represented by the combined inclusion of both MMC_{L4} and MMC_{L5} lesions. Since MMC_{L4} lesions may also (partly) hamper quadriceps muscle innervation, dMUD (calculated as [$MUD_{calf\text{-}muscle}$] - [$MUD_{quadriceps\text{-}mucscle}$]) would be expected to be smaller in MMC_{L4} than MMC_{L5} lesions. Accordingly, we observed smaller dMUD outcomes in MMC_{L4} than MMC_{L5} children. For clinical trials aiming to compare the magnitude of the second hit of spinal damage between different (fetal and neonatal) treatment strategies, this implicates that such comparison may only be performed under level-of-the-lesion matched conditions.[12] All together, in the presently included relatively homogeneous $MMC_{L4\text{-}5}$ group, we observed a significant association between asymmetrically impaired leg muscle function and leg dMUD (representing the magnitude of the 2nd hit of damage). This implicates that the second hit of damage may have an asymmetrical impact upon leg muscle dysfunction. This may be attributed to delivery-related 'secondary' spinal hemorrhages (by the aberrant area vasculosa) and mechanical trauma near different lower motor neuron populations.[1]

We recognize that there are some weaknesses to the study. Firstly, present data are obtained in a select number of SBA children with well-defined $MMC_{L4\text{-}5}$ levels. This selective inclusion was purposely performed to avoid innervation bias by heterogeneous MMC levels. However, we would expect that results would be similar when dMUD calculation would be adapted to a different myotome cranial to the MMC. Secondly, present MUD data were obtained in SBA children with a broad age range (1-18 years of age). In perspective of age independency of MUD outcomes,[8,14] we may assume that the age-range has not affected outcomes.

CONCLUSION

In SBA children between 1 and 18 years of age, age-independent quantitative leg-MUD outcomes are associated with segmental motor and sensory function. These pediatric SBA-MUD data are relevant for the assessment of the segmental neuro-muscular condition during childhood.

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CHAPTER 6

Visual Assessment of Segmental Muscle Ultrasound Images in Spina Bifida Aperta

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ABSTRACT

In spina bifida aperta (SBA), spinal MRI provides a surrogate marker to estimate muscle damage caudal to the myelomeningocele (MMC). This muscle damage by the MMC can be quantified by *intra-individual* comparison of muscle ultrasound density (MUD) caudal versus cranial to the MMC (dMUD = [$MUD_{caudal\text{-}to\text{-}the\text{-}MMC}$] – [$MUD_{cranial\text{-}to\text{-}the\text{-}MMC}$]). Quantitative dMUD assessment requires time, equipment and expertise, whereas it could also be visually determined by differences in muscle echodensity caudal versus cranial to the MMC (visual-dMUD). If visual and quantitative dMUD correspond, visual dMUD assessment could provide a clinical screening parameter.

In 100 SBA muscle ultrasound recordings of patients with various MMC levels, we aimed to compare quantitative dMUD (dMUD = [$MUD_{calf\text{-}muscle/S1}$] – [$MUD_{quadriceps\text{-}muscle/L2\text{-}L4}$]) with visual dMUD assessments by 20 different observers.

Results indicate that quantitative dMUD can be visually detected (sensitivity 86%; specificity 57%), implicating that visual dMUD screening could provide a quick, clinical screening tool for muscle impairment by the MMC.

INTRODUCTION

In spina bifida aperta (SBA), spinal MRI provides a surrogate marker to estimate muscle impairment caudal to the myelomeningocele (MMC). However, due to abnormal segmental innervation caudal to the MMC, actual myopathic changes appear more directly associated with muscle function loss than the MMC demarcation by MRI.[1,2] Such myopathic changes (involving reduction in muscle water content, fat deposition and fibrosis) may induce an increased reflection of the muscle ultrasound beam,[3] resulting in increased muscle ultrasound density (MUD).[2,4-7] As a consequence of the segmental organization of the spinal cord, SBA myotomes caudal to the MMC will be more severely affected than myotomes cranial to the MMC.[8] Such *intra-individual* differences in muscle damage (caudal and cranial to the MMC) are indicated by dMUD (calculated as: dMUD = [$MUD_{caudal\text{-}to\text{-}MMC}$]- [$MUD_{cranial\text{-}to\text{-}MMC}$]). dMUD quantification requires time, equipment and expertise, whereas MUD differences between myotomes caudal and cranial to the MMC could also be visually determined. If quantitative and visual dMUD assessments correspond, visual dMUD assessment would provide a global, fast and easily applicable neuromuscular screening tool.

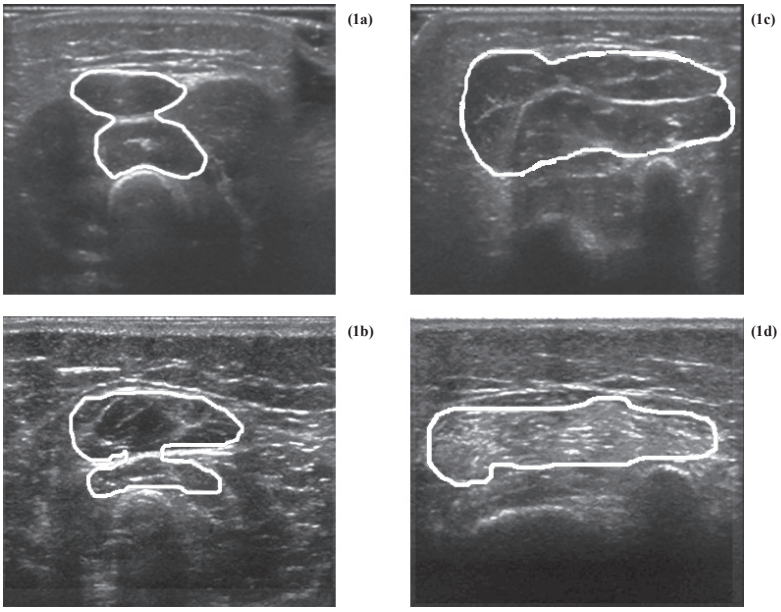
In perspective of the above, our primary aim was to compare quantitative dMUD (i.e. the golden standard) with visual dMUD assessments by diversely skilled neuro-pediatric examiners. If diverse observers could visually assess quantitative dMUD with acceptable sensitivity, visual dMUD assessment could be applicable for clinical screening purposes. Our secondary aim was to evaluate whether quantitative and visual dMUD outcomes change into the same direction under different MMC conditions. This is based upon the reasoning that altered MMC conditions would be expected to have the same impact upon quantitative- and visual- dMUD outcomes. We therefore compared quantitative and visual dMUD between different MMC subgroups, involving: 1. MMC levels cranial or caudal to L_4 and, 2. postnatal age younger or older than three months. The first subgroups are based upon present or absent involvement of the quadriceps muscle (influencing dMUD = [$MUD_{calf\text{-}muscle/S1}$] minus [$MUD_{quadriceps\text{-}muscle/L2\text{-}L4}$]) (for characterization of myotomes, see Ropper and Samuels[9]). In accordance with the "second hit" hypothesis, the latter subgroups are based upon the timing of perinatal damage, which is superimposed upon the congenital neural tube defect (involving perinatal mechanical, chemical and vascular spinal damage[1,10,11]). After a postnatal time-interval of three months, this "second hit" of neural damage would be expected to induce an increase in MUD outcomes caudal to the MMC. In perspective of the above, we thus hypothesized that both MMC levels and postnatal age could influence quantitative and visual dMUD outcomes into the same direction.

In the present SBA study, we thus aimed: 1. to compare quantitative dMUD (i.e. the 'golden standard') with visual dMUD screening results (categorized for different observers with various experience characteristics) and, 2. to compare the forthcoming quantitative and visual MUD results for different underlying MMC conditions.

METHODS

Differential muscle ultrasound density

The medical ethical committee of the University Medical Center Groningen, the Netherlands approved the present study. With informed consent by the parents, we retrospectively included all assessable SBA muscle image sets, which were recorded between 2004 and 2010 in 52 SBA children (median gestational age of 6 (range 0-19) months). In these 52 SBA infants, we obtained all (n=100) SBA muscle image sets of sufficient quality for off-line review (concerning the left and/or right leg). In these 100 MUD data sets, MMC was located: at- or cranial to L_1 (n=2); at L_{2-4} (n=45); at L_5-S_2 (n=50) and caudal to S_2 (n=3) (i.e. median MMC at L_5 [range Th_{11} - S_3]). In accordance with previously described methods,[2,4,5] we assessed MUD of quadriceps (L_2-L_4) and calf muscles (S_1) (figure 1) and quantified dMUD by subtraction. Each SBA MUD set was thus derived from the quadriceps and calf muscles in the same leg in the same SBA infant. All muscle ultrasound recordings were performed with the same ultrasound equipment (*General Electric healthcare logiq 9* ultrasound equipment (Jiangsu, China)) under standardized conditions (for muscle ultrasound gain, dynamic range, compression and time-gain-compensation). We used a linear transducer (14 MHz; gain of 47 dB) and three focal



1. Example of a muscle ultrasound image set obtained in a healthy control and SBA child

Figure 1: Example of a muscle ultrasound image set obtained in a healthy control and SBA child.
Left panels indicate quadriceps muscles (a and c), right panels indicate calf muscles (b and d). Images a and b (upper side of the figure) are obtained in a healthy control child, revealing a similar MUD of quadriceps and calf muscles. Images c and d (lower side of the figure) are obtained in a SBA child (MMC level L5-S1), revealing a higher MUD of the calf muscle than of the quadriceps muscle (i.e. higher echodensity of the calf than quadriceps muscle; quantitative dMUD = 40.1).
SBA = spina bifida aperta; MUD = muscle ultrasound density; MMC = myelomeningocele; L = lumbar; S = sacral; dMUD = difference in muscle ultrasound density between SBA myotomes caudal and cranial to the MMC (i.e. between calf muscle and quadriceps muscle)

points. For standardization purposes and reproducibility by others, we deliberately did not use time gain compensation.[12] An excess of ultrasound gel prevented skin impression. With respect to standardized reference points, we recorded transverse ultrasound images of the quadriceps muscle (probe placed half-way between trochanter major and lateral knee joint cleft) in supine position and of the calf muscle (probe placed at position of maximum circumference) in prone position, which allowed passive muscle relaxation. For digital quantification,[4,5] we stored five ultrasound images per muscle and determined MUD of the quadriceps and calf muscle within a well-defined region of interest (ROI; excluding the surrounding fascia[12]) by Adobe Photoshop (San Jose, CA) [MUD (average pixel value) ranging from 0 (black) to 255 (white)]. The region of interest for the quadriceps muscle consisted of the cross-sectional area of the rectus femoris and vastus intermedius and for the calf muscle of the cross-sectional area of the gastrocnemius and soleus muscle.[12] After exclusion of the highest and the lowest MUD value, we calculated the mean of the three remaining MUD values to minimize variation.

To control for potential segmental MUD differences in healthy controls (i.e. *non*-MMC children), we assessed and compared MUD of quadriceps and calf muscles in 13 healthy control children (age matched with the present SBA study population). In healthy control children [median age 6 (range 0-19) months], MUD- calf was similar to MUD- quadriceps muscle [$MUD_{calf\text{-}muscle}$ vs $MUD_{quadriceps\text{-}muscle}$: 83 (59-106) vs 76 (55-96); medians (ranges); *NS*]. In SBA, we could therefore regard a "positive" dMUD as a pathologic consequence of the MMC (quantified by: $dMUD = [MUD_{calf\text{-}muscle/S1}] - [MUD_{quadriceps\text{-}muscle/L2\text{-}L4}]$).

Observers

For visual dMUD assessment, we recruited 20 observers from the medical staff of the University Medical Center Groningen, the Netherlands. All observers were involved in the assessment and treatment of patients with neuro-muscular disorders and/or neurulation defects (including medical specialists, interns, neuro-pediatric students, technicians and scientists from the departments of pediatrics, neurology, neurophysiology and neuropathology), either with or without experience in myology and/or muscle ultrasound recordings (for characteristics see Table 1). Presence of muscle

Table 1. Self scored muscle-ultrasound (MU) and myology (MY) experience by 20 observers

Group	Number of assessors	Experience in yearsmean (range)
I. Experience in MU and/or MY	10	7.60 (0.25-15)
a- experience in MU, only	4	3.90 (0.25-15)
b- experience in MY, only	4	11.60 (3.00-15)
c- experience in both MU and MY	2	7.25 (0.25-15)
II. No experience in MU nor in MY	10	-

Legend: Observers characterized according to self scored experience in muscle ultrasound (MU) and myology (MY). The "experienced" observer group I (n=10) is subdivided in subgroup Ia (n=4), Ib (n=4) and Ic (n=2).

ultrasound experience was defined by performance and interpretation of muscle ultrasound assessments for more than 3 months (full-time). Presence of myology experience was defined by active and independent clinical participation in the university hospital's neuromuscular team for more than 3 months (involving neurophysiologists, neuropathologists, neurophysiologists and/or pediatric neurologists). All observers were asked to indicate their own experience (with myology and/or muscle ultrasound) anonymously. We checked whether the anonymously self scored experience levels concurred with the pre-estimated experience levels of the total observer group. The percentage of observers *with* experience (in myology and/or in muscle ultrasound) was 50% with an equal distribution among observers with experience in myology and muscle ultrasound (each 25%). The percentage of observers *without* experience (neither in muscle ultrasound, nor in myology) was 50%.

Observers were not informed about clinical data of the SBA infant and were excluded from the preparation of the test slides. In addition to a manual with a written explanation, all observers received a power point file containing 100 different SBA muscle ultrasound image sets for visual dMUD assessment. Each muscle ultrasound image set was presented as a separate test slide, with one image of the quadriceps muscle (left side of the slide) and one image of the calf muscle (right side of the slide). In quadriceps and calf muscles, we encircled the ROI for quantitative and visual dMUD assessment. To avoid inter-observer variation by the delineation of the ROI, we encircled the ROI for all observers. Observers were instructed to indicate whether MUD within the ROI of the calf muscle (indicated on the right) was higher than MUD within the ROI of the quadriceps muscle (indicated on the left), or not. Observers were not allowed to consider other muscle characteristics than muscle echogenicity within the indicated ROI. All visual scores were subdivided according to the self scored experience in myology or muscle ultrasound.

Since dMUD has to be substantial enough to allow visual discrimination, we firstly assessed the smallest dMUD (dMUD cut-off point) at which a quantitative dMUD can be visually recognized with a sensitivity exceeding 80% (i.e. the pre-defined optimum cut-off point). We therefore calculated mean sensitivity and specificity of visual dMUD for incremental dMUD cut off values (i.e. for 0-5; 5-10; 10-15, etc.).

Secondary Aim

For the secondary aim, we categorized all image sets according to MMC levels (cranial and caudal to L_4) and postnatal age (0-3 vs > 3 months postnatal age). In all infants, the upper border of the MMC was indicated by neonatal spinal MRI. Quantitative- and visual- dMUD outcomes were subsequently compared for the above described MMC conditions.

Statistics

We performed statistical analysis with SPSS 16.0 for Windows. For the visual recognition of quantitative dMUD outcomes by 20 observers, we determined the sensitivity and specificity for all

possible cut off points. Results were compiled into a receiver operating characteristic (ROC) curve. We subsequently determined the optimal quantitative dMUD cut off point at which the sensitivity of visual recognition of dMUD exceeded 80%.

Sensitivity and specificity of visual dMUD recognition was normally distributed, as shown by Shapiro Wilk test. We compared sensitivity and specificity of visual dMUD recognition for experienced and inexperienced observer groups by the student T-test. Inter-observer agreement was assessed by Cohen Kappa's test. An acceptable range for inter-observer agreement was pre-defined as 0.40 to 0.60.[13,14] We subdivided all muscle ultrasound images in accordance with radiologic MMC levels and age. As shown by the Shapiro Wilk test, MMC levels and age were not normally distributed. We therefore assessed and compared (quantitative and visual) dMUD outcomes for MMC levels (cranial or caudal to L_4) and for age (younger versus older than 3 months) by Mann Whitney test. Statistical significance was set at a $p<.05$.

RESULTS

I. Quantitative vs Visual dMUD assessment by three observer groups:

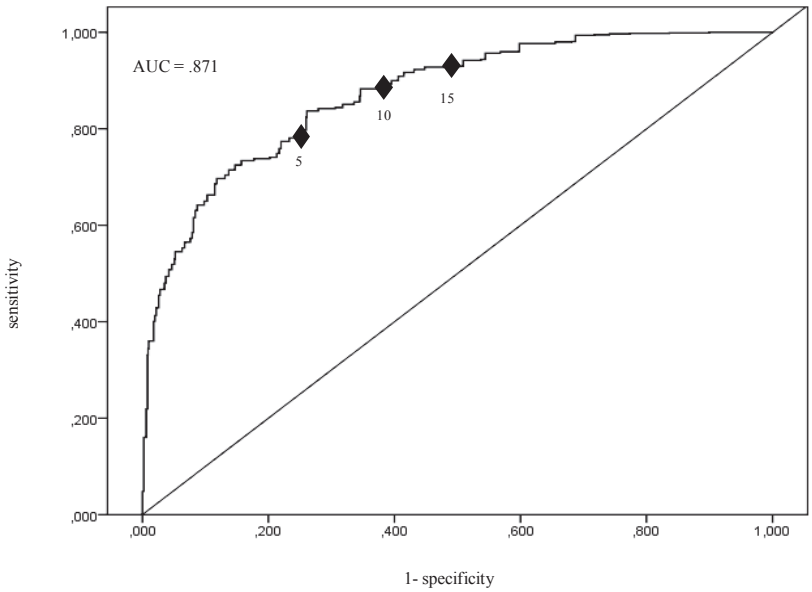
Mean sensitivity and specificity for all dMUD cut-off points are shown in Table 2. The ROC curve for visual dMUD assessment is shown in figure 2. Visual dMUD recognition discriminated a quantitative dMUD of 10 grey-values with a sensitivity of 86% and specificity of 57%. The percentage of false negatives was 13%. The area-under-the-curve was .871.

The collected outcomes of all self-scored observer-experience levels resembled pre-estimated experience levels of the whole group (50% had experience in muscle ultrasound and/or myology; 50% had no experience), for further subdivision into observer categories, see Table 1. Comparing visual dMUD outcomes between observer categories revealed a lower sensitivity for myology-

Table 2. Sensitivity and Specificity of dMUD cut off points

Cut off points (in grey values)	Mean sensitivity (in %)	Mean specificity (in %)
> 0	74.3	87.7
> 5	80.4	76.0
> 10	86.1	56.9
> 15	91.5	54.9
> 20	96.6	45.6
> 25	98.6	42.0
> 30	98.6	36.9
> 35	100	35.9
> 40	100	35.2
> 45	100	33.6

Legend: Visual dMUD scores by 20 assessors. Cut off points indicate mean sensitivity and specificity for incremental dMUD values (ranging from 0 to 45). dMUD = $[MUD_{calf\text{-}muscle}] - [MUD_{quadriceps\text{-}muscle}]$.



2. ROC-curve of visual dMUD assessment

Figure 2: ROC-curve of visual dMUD assessment
The x-axis indicates 1- specificity (false positive rate) for the visual dMUD recognition. The y-axis indicates the sensitivity for the visual dMUD recognition. The inserted dots (in the curve) represent a dMUD cut-off point of 5, 10 and 15 grey values. At the cut off point of 10 grey values, sensitivity of 86% has exceeded the 80% level. The area under the curve for visual dMUD recognition is .871.
ROC-curve = receiver operating characteristic-curve; dMUD = difference in muscle ultrasound density

experienced than for myology-inexperienced groups (*p=.012*), see Table 3. At a cut-off point of 10 grey values, inter-observer agreement was 0.514. Sub-division in inexperienced, muscle ultrasound-experienced, and myology-experienced observer subgroups revealed similar kappa scores (0.536; 0.541 and 0.459; resp. (*NS*)), which were all three within the pre-defined acceptable range for kappa scores (0.40 to 0.60).

II. The relation between dMUD and various MMC conditions (MMC level and age):

Associating quantitative dMUD with MMC levels revealed higher dMUD outcomes for MMC levels caudal than cranial to L_4 (*p=.032)*. The sensitivity of visual dMUD recognition (i.e. percentages of assessors discerning a positive dMUD revealed also higher outcomes for MMC levels caudal than cranial to L_4 (*p=.005)*. Associating postnatal age subgroups (younger versus older than 3 months of age) with included MMC levels, did not reveal significant differences (*p=.857)*. Quantitative dMUD assessment revealed higher outcomes after- than before- the third month of postnatal life (*p=.008)*. Accordingly, sensitivity of visual dMUD recognition (i.e. percentages of assessors discerning a positive dMUD) was also higher after- than before- the third month of postnatal life (*p=.027)*.

Table 3. Sensitivity and specificity of visual dMUD recognition

		Total Group			Myology			Muscle ultrasound		
		experience		T-test†	experience		T-test†	experience		T-test†
	All N=20	yes N = 10	no N = 10		yes N = 6	no N = 14		yes N = 6	no N = 14	
Sensitivity	86.3%	83.3%	89.3%	.182	78.7%	89.2%	.012	84.2%	87.2%	.550
Sensitivity	59.6%	62.4%	56.9%	.330	57.0%	65.9%	.140	63.5%	58.0%	.371

Legend: Sensitivity and specificity of visual dMUD recognition (at the optimal cut off point of 10 grey-values) according to the observers' experience. There were no significant differences between the combined experienced and inexperienced observer groups. Observers who were more experienced in myology had a lower sensitivity. dMUD = [$MUD_{calf\text{-}muscle}$] – [$MUD_{quadriceps\text{-}muscle}$]. † two-tailed test; p-values are indicated.

DISCUSSION

In children with SBA, we aimed to associate *intra*-individual visual and quantitative dMUD outcomes. Independent of the observer characteristics, results indicated a positive relationship between visual and quantitative dMUD outcomes. Furthermore, both quantitative and visual dMUD parameters were changed into the same direction by different MMC conditions (MMC level and postnatal age). Present SBA data may implicate that visual dMUD could provide a simple and quick screening method to estimate potential muscle damage caudal to the MMC.

In pediatric studies, quantitative MUD assessment has been described as a reliable, non-invasive technique for the detection of neuromuscular disease.[4-7,15] However, quantitative MUD assessment requires time, equipment and expertise, which may limit general application. In this perspective, it was hypothesized that visual MUD assessment could provide an easier assessable screening tool.[12,16,17] Accordingly, it was previously shown that visual muscle ultrasound assessment could discern Heckmatt's scores, but with a lower sensitivity than quantitative MUD assessment (71% and 87%, for visual and quantitative assessment, respectively[12]). In the present SBA MUD study, we therefore applied a more simplified visual screening method involving discrimination between *intra*-individual MUD differences (dMUD). Results indicated that a MUD difference of at least 10 grey-values could be visually discerned with a sensitivity exceeding 80%. Applying this optimum dMUD cut-off point (of 10 grey-values) for visual dMUD outcomes, revealed an acceptable accuracy (81%) and sensitivity (86%) of dMUD detection, whereas specificity (57%) appeared low. These characteristics are typical for a 'rule out test', implicating that if a positive quantitative dMUD is present, it can be visually discerned in 86% of the cases.

Conversely, a negative visual dMUD screening outcome would make a positive quantitative dMUD outcome unlikely (i.e. false negative rate is 13%). In SBA children, this implicates that intra-individual visual dMUD assessment could provide a quick and non-invasive screening method for segmental

muscle damage. However, in case of an unexpected visual dMUD outcome, quantitative dMUD confirmation (i.e. golden standard) can be advisory.

Interestingly, inexperienced and muscle ultrasound experienced observers achieved higher sensitivities than myology-experienced observers. Since the self scored and pre-estimated experience characteristics did not differ, we can not attribute this finding to falsely self-scored experience levels. This may implicate that a simplified test (involving discrimination between grey-values within a pre-defined ROI) rather than a complex test (such as qualitative visual interpretation involving Heckmatt scale), could exert a more favorable effect upon the outcomes by the inexperienced than by mycology- experienced observers. Since the ROI was clearly indicated to all observers, and since the observers were not allowed to evaluate any other aspects than MUD within the ROI, these outcomes do not necessarily implicate that inexperienced observers are technically more capable of visual dMUD screening. Especially when visual MUD assessment would be applied for more complex and diverse neuromuscular diseases (requiring qualitative interpretation of the image), we would expect that both myology- and muscle ultrasound- experienced observers would achieve better results than inexperienced observers.

Finally, we observed that both quantitative and visual dMUD screening outcomes change into the same direction according to the segmental MMC location and postnatal age of the child (i.e. the time period to induce maximal histological muscle alterations). The latter age-dependent finding appears reflective of the consequences by the second hit hypothesis, involving ongoing perinatal spinal trauma (inducing secondary muscle denervation damage and subsequently delayed muscle fibrosis and muscle fat deposition, thereafter[11]).

All together, in children with SBA we conclude that visual dMUD assessment could provide a simple screening method for a quick estimation of segmental muscle damage.

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CHAPTER 7

Fetal Endoscopic Myelomeningocele Closure Preserves Segmental Neurological Function

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ABSTRACT

Aim: Our aim was to compare the effect of prenatal endoscopic with postnatal myelomeningocele closure (fetally operated spina bifida aperta [fSBA]) versus neonatally operated spina bifida aperta [nSBA]) on segmental neurological leg condition.

Method: Between 2003 and 2009, the fetal surgical team (Department of Obstetrics, University of Bonn, Germany) performed 19 fetal endoscopic procedures. Three procedures resulted in fetal death, three procedures were interrupted by iatrogenic hemorrhages and 13 procedures were successful. We matched each successfully treated fSBA infant with another nSBA infant of the same age and level of lesion, resulting in 13 matched pairs (mean age 14mo; SD 16mo; f/m = 1.6; female-16, male-10). Matched fSBA and nSBA pairs were compared in terms of segmental neurological function and leg muscle ultrasound density (MUD). We also determined intraindividual difference in MUD (dMUD) between myotomes caudal and cranial to the myelomeningocele (reflecting neuromuscular damage by the myelomeningocele) and compared dMUD between fSBA and nSBA infants. Finally, we correlated dMUD with segmental neurological function.

Results: We found that, on average, the fSBA group were born at a lower gestational age than the nSBA group (median 32wks [range 25–34wks] vs 39wks [34–41wks]; p=0.001) and experienced more complications (chorioamnionitis, premature rupture of the amniotic membranes, oligohydramnios, and infant respiratory distress syndrome necessitating intermittent positive-pressure ventilation). Neurological function was better preserved after fSBA than after nSBA (median motor and sensory gain of two segments; better preserved knee-jerk [p=0.006] and anal [p=0.032] reflexes). The dMUD was smaller in fSBA than in nSBA infants (mean difference 24, 95% confidence interval [CI] 15–33; p<0.05), which was associated with better preserved segmental muscle function.

Interpretation: Fetal endoscopic surgery is associated with spinal segmental neuroprotection, but it results in more complications. Before considering clinical implementation of fetal endoscopic myelomeningocele closure as standard care, the frequency of complications should be appropriately reduced and results assessed in larger groups over a longer period of time.

INTRODUCTION

In fetuses with spina bifida aperta (SBA), leg movements caudal to the myelomeningocele (MMC) are often present, but they disappear shortly after birth.[1–4] Fetal neuroprotection strategies (such as fetal MMC closure) aim to maintain leg motor function by preserving neuromuscular innervation.[5–9] In humans, fetal closure of the MMC is performed by open[7] or endoscopic surgical techniques.[8,9] Recently published results from the Management of Myelomeningocele Study,[7,9] a randomized controlled trial, suggest that open fetal surgery can improve neurological outcome. However, open fetal surgery is also associated with fetal and maternal risks, including preterm birth, intraoperative complications, and uterine scar defects.[7,9] One of the major goals of fetal endoscopic MMC closure is to preserve neuromuscular integrity and to minimize iatrogenic damage.[8] Kohl et al.[8] reported that fetal endoscopic treatment resulted in an improved neurological condition, although effects on leg motor function were incompletely assessed. From a theoretical perspective, fetal endoscopic therapy might induce neurological gain by improving both cerebral (drain dependence; Chiari 2 malformation) and spinal (MMC) conditions.[10] However, given the presence of many aberrant, readily haemorrhaging, blood vessels in the region of the MMC, it remains a matter of debate whether fetal endoscopic MMC closure can protect neuromuscular innervation at and caudal to the MMC.[11] In the present study, we aimed to elucidate the effect of fetal endoscopic MMC closure on segmental neurological function and integrity caudal to the MMC.

Recently advanced magnetic resonance imaging (MRI) techniques have provided clinicians with new diagnostic insights (into muscle structure, function, and metabolism) without the need for invasive biopsy.[12] To avoid anaesthesia in young children, neuropaediatric clinicians can also apply the non-invasive 'muscle ultrasound' technique, which measures quantitative muscle ultrasound density (MUD) parameters for neuromuscular assessment and surveillance.[13,14] MUD parameters are based upon secondary muscle alterations after neural innervation damage (inducing reduced muscle water content, fibrosis, fat deposition, and atrophy), causing increased MUD.[15] From this perspective, we reasoned that muscle ultrasonography could provide a useful, quantitative, non-invasive tool in children with SBA. In particular, the intraindividual difference in MUD (dMUD) between myotomes caudal and cranial to the MMC could quantitatively reveal muscle damage caused by the MMC itself.[14] In the present study, we compared neurological and quantitative MUD parameters in children with SBA treated by fetal endoscopic MMC closure (fetally operated SBA [fSBA]) or neonatal MMC closure (neonatally operated SBA [nSBA]).

METHOD

Participants

The medical ethics committees of Bonn University, Germany, and the University Medical Center Groningen (UMCG), the Netherlands, approved the comparison of age- and lesion-matched fSBA and nSBA children. fSBA children were operated on and delivered at Bonn University; nSBA children were operated on and treated at the UMCG. Both participating university centres provided

multidisciplinary care by spina bifida teams. The UMCG SBA team caring for the nSBA control group is associated with similar neonatal predictive parameters[3] and neurological outcomes[16] as reported by Hunt and Poulton.[17] All parents of children with SBA included in the study (mean age 14mo; SD 16mo; mean upper MMC level at L_3; SD one segment; female-16, male-10) gave informed consent. Between 2003 and 2009, Kohl's surgical team performed a total of 19 fetal endoscopic procedures at the German Center for Fetal Surgery and Minimally-Invasive Therapy, Bonn University, Germany. For technical details of the surgical patch coverage, refer to the descriptions published by the operating team.[8,18] Of the 19 fetuses who underwent endoscopic procedures, 13 infants survived and were successfully treated, and all 13 were included in the study. Three fSBA fetuses died (from complicated anaesthesia, placental haemorrhaging, or oligohydramnios-related lung hypoplasia; for further information see Kohl et al.[18]) and in three cases the endoscopic procedure was interrupted by placental haemorrhaging. These three fetuses survived and underwent postnatal MMC closure. All fetal endoscopic procedures were performed at a gestational age of between 20^{+5} and 24^{+3} (median 23^{+0}) weeks.

We matched each fSBA infant with another of the same age and with the same level of lesion, resulting in 13 matched pairs. The level of the lesion was considered to be the upper border of the MMC (determined by fetal ultrasonography and confirmed by postnatal MRI). When more than one lesion-matched nSBA child was available, we selected the infant nearest in age. Matched pairs showed age ranges from 0 to 2 months under 1 year of age and from 0 to 1 year in older children. All 13 nSBA comparison children were born at the UMCG and operated on during the first week of life. The data for each individual are shown in Table 1.

All fSBA children were born by Caesarean section (performed after initiation of preterm labour). All nSBA children were born by vaginal delivery. To study the potential influence by Caesarean section, we included another 13 age- and lesion-matched pairs of nSBA children delivered by Caesarean section or vaginal delivery (mean upper MMC level at L_4; SD one segment; mean age 29mo; SD 24mo). In pregnancies in which the child with SBA underwent neonatal operation, Caesarean section was performed either electively (n=9) or after initiation of labour (failed delivery progression; n=4). nSBA children delivered by Caesarean section were born and treated at Bonn University and Cologne Children's Hospital; all nSBA children who were delivered vaginally were born and treated at the UMCG.

Neurological examination

Standardized neurological examinations were performed by the same paediatric neurologist. Neurological examinations were videotaped and scored offline for segmental neurological (motor and sensory) assessment. Motor levels were indicated by the lowest myotomes involved in active motor behaviour. Sensory levels were indicated by the lowest dermatome at which a pinprick elicited an emotional response. In children in whom neurological levels were different on the left and right sides, we obtained the mean segmental level of both legs. For statistical comparison between age-

Table I: Individual data of included fetally-operated and neonatally-operated spina bifida aperta in aged and lesion-matched pairs of children

Pair	Matched MMC level	Age at assessment	Other spinal pathology	Cerebral malformation	Shunt dependence	Infantile complications
1a	T12	2y	TC	Chiari 2	+	IRDS, E
1b	T12	1y	–	Chiari 2	+	–
2a	L2	1y	–	Chiari 2	–	IRDS, E, A, LH, PPHN, I
2b	L2	1y	–	Chiari 2, CCH	+	–
3a	L3	0mo	MLC	Chiari 2	–	IRDS
3b	L3	0mo	Syrinx	Chiari 2	+	–
4a	L3	1mo	–	Chiari 2, SPA	–	IRDS
4b	L3	0mo	–	Chiari 2	+	–
5a	L3	2y	DM	Chiari 2, SPA	+	IRDS, I
5b	L3	3y	–	Chiari 2	+	–
6a	L3	1y	TC	Chiari 2, SPA	–	IRDS
6b	L3	2y	Syrinx	Chiari 2	+	–
7a	L3	0mo	–	Chiari 2	–	IRDS, S
7b	L3	0mo	–	Chiari 2, CCH	+	–
8a	L4	2mo	–	–	–	IRDS
8b	L4	0mo	TC, syrinx,	Ch-2	+	–
9a	L4	5mo	–	–	–	–
9b	L4	3mo	Syrinx	Chiari 2	+	–
10a	L4	5y	TC	Chiari 2, CCH, MC	+	IRDS, E, I
10b	L4	4y	TC, syrinx	Chiari 2	–	–
11a	L4	3y	–	Chiari 2	–	IRDS, E, PPHN, I, S
11b	L4	3y	Syrinx	Chiari 2, CCH	+	–
12a	L5	1y	TC	Chiari 2, CCH, SPA	+	IRDS
12b	L5	1y	–	Chiari 2	+	–
13a	L5	0mo	TC	Chiari 2	–	IRDS, LH, PPHN, A
13b	L5	0mo	–	Chiari 2	+	–

a, fetally operated and Caesarean section; b, neonatally operated and vaginal delivery; MMC, myelomeningocele; Th, thoracal; L, lumbar; +, present; –, absent; TC, tethered cord; MLC, myelum cyst; DM, diastemomyelia; CCH, corpus callosum hypoplasia; SPA, septum pellucidum agenesis; MC, microencephaly; IRDS, infant respiratory distress syndrome; E, endocrine disturbance; A, asphyxia; LH, lung hypoplasia; PPHN, persistent pulmonary hypertension; I, neonatal infection; S, sepsis.

and lesion-matched pairs, we attributed numerical scores to each neurological level ranging from 0 to 8 (i.e. T_{12}=0; L_1=1; L_2=2; L_3=3; L_4=4; L_5=5; S_1=6; S_2=7; and no neurological dysfunction=8).

For analysis of leg reflex activity, we examined knee-jerk (L_{2-4}) and anal reflexes (S_{3-5}). Knee-jerk reflexes were evoked in the supine position. The reflex was scored as present when at least five taps upon the tendon evoked a visible contraction of the quadriceps muscle and as absent when no contraction was observed. We attributed a score of '2' to visible reflexes in both legs; '1' to a visible reflex in one leg; and '0' to lack of visible reflexes. The anal reflex was evoked in the prone position and scored offline as present (visible sphincter contractions at both anal sides: '2' points), weak (sphincter contractions at one side: '1' point), or absent (no contractions: '0' points). We compared scores between age- and lesion-matched fSBA and nSBA groups. As the Achilles' tendon reflex is not consistently present in healthy neonates and infants,[19] we excluded it from the analysis.

Assessment of muscle ultrasound density

Muscle ultrasound registrations of biceps, quadriceps, and calf muscles were assessed with standard muscle ultrasound gain, dynamic range, compression, and time-gain compensation parameters.[15] In accordance with standardized reference points, biceps and quadriceps muscle ultrasound images were recorded in the supine position and calf muscles in the prone position. For digital quantification, we stored five ultrasound images per muscle and determined MUD within a well-defined region of interest. MUD outcome is derived by excluding the highest and lowest values and calculating the mean of the three remaining MUD values. To minimize variation and bias, all muscle ultrasound recordings were performed by the same investigators (RJV and JHvdH). In Germany, recordings were performed with portable ultrasound equipment (LOGIQ e; GE Healthcare; Jiangsu; China). In the Netherlands, muscle ultrasound recordings were performed with fixed ultrasound equipment (LOGIQ 9; GE Healthcare). Portable and fixed muscle ultrasound machines were compatible GE Healthcare LOGIQ systems, both owned by UMCG. Both machines were formally calibrated by the GE technician before the study. Before assessments, we compared the MUD outcomes of both machines by performing a regression. We therefore assessed MUD in leg myotomes of healthy children and children with SBA (*n*=32; age range 3–64mo; myotomes of C_5–C_6 [biceps muscle], L_2–L_4 [quadriceps muscle], and S_1–S_2 [calf muscle], i.e. both cranial and caudal to MMC). Thus, the same investigator separately assessed these myotomes twice using each ultrasound machine. The MUD conversion equation is given as:

$$MUD_{logiq\ 9}=37.262 + 1.368 * MUD_{logiq\ e}\ [r^2=0.74].$$

This conversion equation is reliable, as indicated by the fact that coefficients of variation were similar for both machines (LOGIQ 9, 24%; LOGIQ e, 20%) and the Bland–Altman plot showed no residual correlation. Evaluation of MUD within the bounds of error of the conversion equation revealed similar results.

Inter- and intraindividual comparison of muscle ultrasound density parameters

Of the 13 age- and lesion-matched pairs of infants, 12 had a lumbar MMC and one pair had a thoracic MMC. In all 12 pairs with a lumbar MMC (pairs 2–13), the quadriceps muscle was innervated at or cranial to the MMC and the calf muscle caudal to the MMC. In these 12 pairs, we assessed the MUD of the quadriceps (innervation L_2–L_4, i.e. at/cranial to the MMC) and calf muscles (innervation S_1–S_2, i.e. caudal to the MMC) and determined the intraindividual MUD difference [i.e. dMUD=($MUD_{calf\ muscle}$) – ($MUD_{quadriceps\ muscle}$)]. In the only thoracic MMC pair (pair 1), we computed dMUD between biceps (C_5–C_6, i.e. cranial to the MMC) and calf muscles (S_1–S_2, i.e. caudal to the MMC) by dMUD=($MUD_{calf\ muscle}$)–($MUD_{biceps\ muscle}$). We estimated the functional significance of the dMUD treatment outcome by associating dMUD with neurological segmental S_1 function (caudal to the MMC).

Statistical analysis

Statistical analysis was performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). As MUD values were not normally distributed (according to Q–Q plots and the Shapiro-Wilk test), we compared matched pairs by non-parametric Wilcoxon signed-rank test. To obtain an estimate of the functional significance of the quantitative MUD treatment effect, we associated MUD outcomes with segmental neurological function using the Mann–Whitney *U* test. The level of significance was α=0.05.

RESULTS

Clinical data

Clinical data are indicated in Table I. fSBA children were delivered at a lower gestational age than nSBA children (median 32wks [range 25^{+3}–34^{+3}wks] vs 39wks [34^{+6}–41^{+2}wks], respectively; p=0.001). The prevalence of shunt-dependent hydrocephalus was lower in fSBA than in nSBA children (4/13 vs 12/13, respectively; p<0.05). Complications of fetal endoscopic surgery included amnion infection (3/13 pregnancies), maternal haemorrhaging (3/13 pregnancies), premature rupture of the amniotic membranes (11/13 pregnancies), and oligohydramnios (8/13 pregnancies). All fSBA neonates received respiratory support (10/13 neonates by intermittent positive-pressure ventilation; 13/13 neonates by continuous positive airway pressure). Perinatal complications consisted of asphyxia (2/13 neonates), infant respiratory distress syndrome (12/13 neonates), lung hypoplasia (2/13 neonates), infections (7/13 neonates), and endocrine disturbances (4/13 neonates).

Neurological outcomes

Comparison of age- and lesion-matched fSBA and nSBA children revealed better preserved neuromuscular function in fSBA than in nSBA children (i.e. a median difference of two myotomes [range –0.5 to 4] for motor function and two dermatomes [range –1.5 to 5] for sensory function; p=0.008 and p=0.003, respectively; see Figs. 1 and 2).

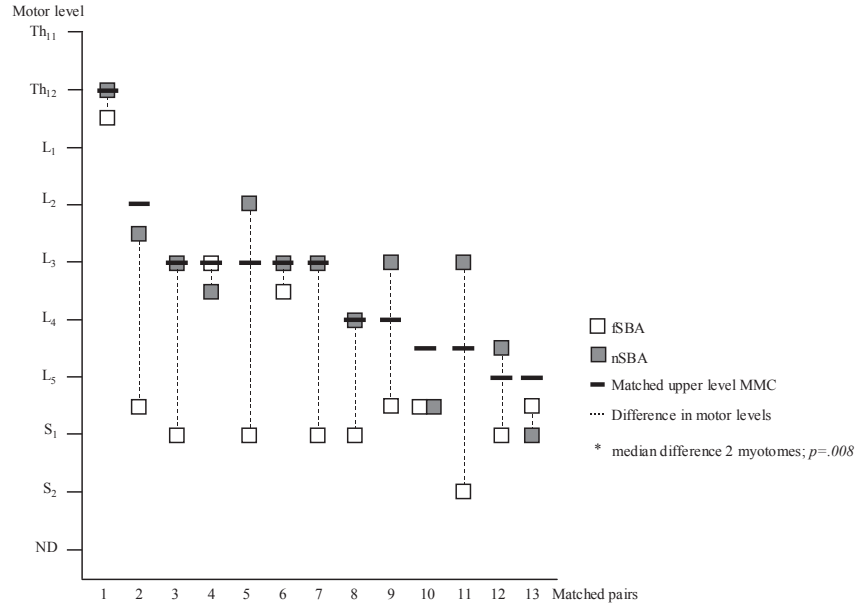

Figure 1: Segmental motor function in age- and lesion-matched pairs of fSBA- and nSBA children. The y-axis indicates the highest myotome participating in spontaneous movements. Segmental motor function is better preserved in fSBA- than in nSBA children (median difference: two myotomes; p=0.008). fSBA, fetally operated spina bifida aperta; nSBA, neonatally operated spina bifida aperta; ND, no deficit.

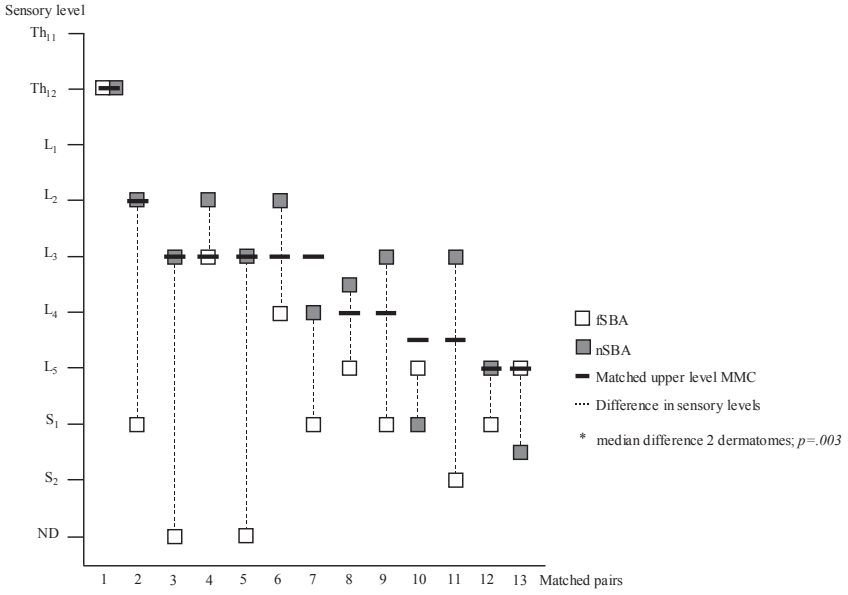

Figure 2: Segmental sensory function in age- and lesion-matched pairs of fSBA and nSBA children. The y-axis indicates the highest dermatome with sensory function. Sensory function is better preserved in fSBA than in nSBA children (median difference: two dermatomes; p=0.003). fSBA, fetally operated spina bifida aperta; nSBA, neonatally operated spina bifida aperta; ND, no deficit.

Numerical scores for both knee-jerk and anal reflexes were higher in fSBA than in lesion-matched nSBA infants (knee-jerk reflexes: 22/26 vs 7/26 points, respectively; p=0.006; anal reflexes: 11/26 vs 0/26 points, respectively, p=0.032).

Inter- and intraindividual comparison of muscle ultrasound density parameters

Comparison of MUD cranial to the MMC (i.e. $MUD_{quadriceps\ muscle}$) did not reveal significant differences between age- and lesion-matched fSBA and nSBA children (mean difference 15; 95% CI 5–24). Comparison of MUD caudal to the MMC (i.e. $MUD_{calf\ muscle}$) showed that the lowest outcome group was fSBA children (mean difference 20; 95% CI 7–34; p<0.05). To estimate the functional significance of this finding, we subsequently associated $MUD_{calf\ muscle}$ with segmental neurological S_1 functioning. Lower fSBA $MUD_{calf\ muscle}$ outcomes were associated with preserved neurological S_1 (motor and sensory) function ($MUD_{calf\ muscle}$ in present vs absent plantar flexion: mean difference 31; 95% CI 4-58; and $MUD_{calf\ muscle}$ in present vs absent sensory S_1 function: mean difference 31; 95% CI 5–58; both p<0.05; see Fig. 3a,b). As preserved neurological S_1 function could theoretically be attributed to better preserved cerebral and spinal conditions, we determined the dMUD in each infant. Mean dMUD was lower in the fSBA group than in the nSBA group (mean difference 24; 95% CI 15–33; p<0.05). To estimate the functional significance of this finding, we associated dMUD with neurological S_1 function (i.e. caudal to the MMC). Quantitative fSBA dMUD outcomes appeared to be related to segmental neurological S_1 functioning (dMUD in present vs absent plantar flexion: mean difference 20; 95% CI –7-47; cut-off point 40; p<0.05; dMUD in present vs absent sensory S_1 function: mean difference 32; 95% CI 12–52; both p<0.05; Fig. 3c).

Effect of delivery mode

Neurological comparison of motor and sensory function between nSBA children born by Caesarean section and nSBA children born by vaginal delivery revealed no significant differences. The knee-jerk and anal reflex response pattern was similar in both groups. Age- and lesion-matched $MUD_{calf\ muscle}$ (caudal to the MMC) and dMUD did not significantly differ between the two groups (MUD mean difference 26; 95% CI 14–38; dMUD mean difference 21; 95% CI 10–31).

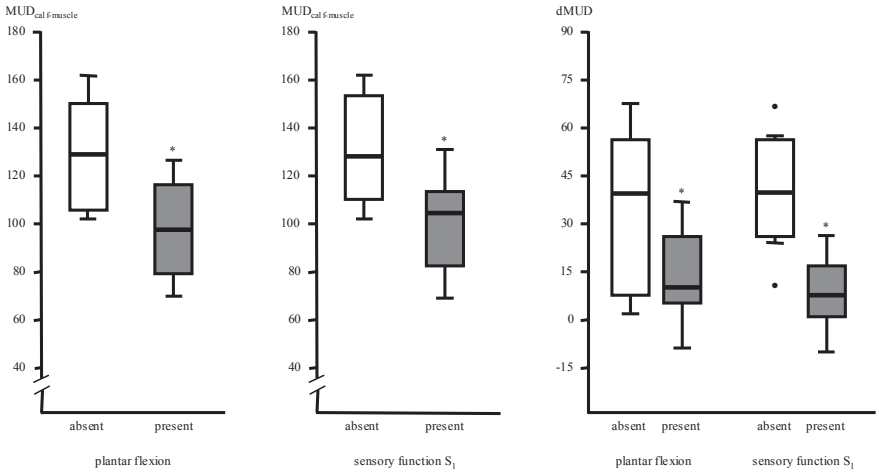

Figure 3: Muscle ultrasound density (MUD) parameters*. (a) Association between fSBA $MUD_{calf\ muscle}$ and calf muscle function. The calf muscle is innervated caudal to the MMC (S1–S2). The x-axis subdivides motor function into present and absent foot plantar flexion (calf muscle function). The y-axis indicates $MUD_{calf\ muscle}$. In fSBA, $MUD_{calf\ muscle}$ is associated with calf muscle function (p=0.047). (b) Association between fSBA $MUD_{calf\ muscle}$ and sensory S1 function. The x-axis subdivides sensory function into cranial and caudal to S1. The y-axis indicates $MUD_{calf-\ muscle}$. In fSBA, $MUD_{calf\ muscle}$ is associated with sensory S1 function (p=0.037). (c) Comparison between fSBA dMUD and segmental neurological (motor and sensory) function caudal to the MMC. The x-axis represents S1 motor function (present or absent plantar flexion) on the left and S1 sensory function (present or absent perception) on the right. The y-axis indicates intraindividual dMUD. Quantitative dMUD appeared associated with segmental neurological (motor and sensory) function caudal to the MMC. *Box plots mark first and third quartiles; whiskers represent data points 1.5 times the interquartile range below and above the first and third quartiles. Dots represent outliers. $MUD_{calf\ muscle}$, muscle ultrasound density of calf muscle; fSBA, fetally operated spina bifida aperta; S, sacral segment; dMUD, intraindividual difference in muscle ultrasound density.

DISCUSSION

In the present study, we aimed to elucidate whether fetal endoscopic MMC closure can provide fetal spinal neuroprotection. Comparison of age- and lesion-matched infants revealed that segmental neurological outcomes and muscle ultrasound densities were better after fetal endoscopic MMC closure than after neonatal MMC closure.

Fetal closure of MMC can be performed by open[7,9] or endoscopic surgical techniques.[8,10,18] The recently published results of the Management of Myelomeningocele Study have convincingly shown that open fetal surgery can improve neurological outcome.[7,9] However, open fetal treatment is also associated with iatrogenic maternal and fetal risks, which should be taken into account.[7,9,10] As with open fetal treatment, we observed severe iatrogenic complications after endoscopic fetal treatment, such as premature rupture of the amniotic membranes, amnion infection, oligohydramnios, preterm delivery, pulmonary hypoplasia, and fetal death.[18] Despite iatrogenic complications, fetal endoscopic MMC closure is associated with preserved cerebral condition (i.e. ameliorated Chiari 2

malformation and reduced drain dependence).[18] Accordingly, our results reveal a reduced incidence of drain dependence similar to that reported by the Management of Myelomeningocele Study.[7] However, as the fSBA children were young (median age 14mo), a longer follow-up period might be needed to confirm whether these differences are persistent.[9]

The aim of the present study was to determine whether fetal endoscopic surgery can preserve segmental leg function by providing spinal neuroprotection. Our data indicate that segmental leg function is better preserved after fSBA than after nSBA treatment (median of two segments motor and sensory median). As segmental reflexes cranial and caudal to the MMC (i.e. knee-jerk and anal reflex activity) were also better preserved in fSBA than in nSBA, both cerebral and spinal improvements could (theoretically) contribute to these results. For further differentiation, we assessed dMUD and compared the outcomes in age- and lesion-matched fSBA and nSBA children. The results indicate lower dMUD and better preserved segmental neurological (motor and/or sensory) function caudal to the MMC in fSBA than in nSBA children. Thus, these data may indicate that spinal neuroprotection is (at least partly) involved. It could also be argued that results can be attributed to group differences in rehabilitation practice. However, as preserved fSBA outcomes were already present in neonates, and as all nSBA children received multidisciplinary care by a large, well-equipped academic spina bifida team (providing care for the northern to middle eastern part of the Netherlands, reporting outcomes within the expected academic European range),[3,16,17] whereas fSBA children received care at different local European centres, this appears less likely. Taken together, the present data appear supportive of the 'second-hit hypothesis', suggesting that segmental neurological damage at the MMC[20] is partly ameliorated by fetal endoscopic spinal neuroprotection.

It is known that SBA pregnancies with predefined conditions (i.e. midlumbar cystic MMC) may neurologically benefit from elective Caesarean section (before the onset of uterine contractions).[21,22] It might therefore be questioned whether group differences in delivery modes (Caesarean section vs vaginal delivery) could explain the present results. However, as Caesarean section of fSBA children was performed *after preterm initiation of delivery* and because all cystic fSBA MMCs had been operated on *before* Caesarean section, this treatment did not correspond with predefined 'favourable' Caesarean section conditions. To control for a (theoretic potentially) confounding effect by the delivery mode under 'unfavourable Caesarean section conditions', we assembled a second lesion- and age-matched group of nSBA children delivered by Caesarean section and nSBA children delivered vaginally, regardless of delivery initiation and regardless of MMC type. Under such 'unfavourable' conditions, we did not detect a confounding influence by Caesarean section. Although we conclude that Caesarean section (under 'unfavourable' conditions) did not confound the current results, one cannot extrapolate forthcoming data to the assumption that Caesarean section is thus ineffective when it is *electively* performed for *midlumbar, cystic MMC*.[21,23]

We realize that the present study has several limitations. First, the number of fetal endoscopic surgeries assessed in this study is small. However, we included all feasible fSBA children and therefore our results could be regarded as indicative. Second, neurological assessments were

performed with prior knowledge of the treatment groups. However, all assessments and scorings were performed by the same UMCG team, which has been critical about fetal surgery and never performed, collaborated, or referred children with SBA for fetal MMC closure. Furthermore, this limitation did not influence quantitative MUD outcomes, which substantiated neurological results. Third, the children with SBA were relatively young (median age 14mo). However, a longer period of neurological surveillance is needed to control for the potential occurrence of delayed complications (shunt dependency or tethering) and to confirm whether more preserved neurological fSBA outcomes persist.[9] Fourth, there was still a small difference in age between members of matched pairs. However, as MUD is independent of age,[24] we do not expect that this influenced outcomes. Fifth, as our sample included only one matched pair with a thoracic MMC, the present results are mainly representative for individuals with lumbar MMC (which is associated with a distinctly different prognosis and life expectancy from thoracic MMC).[25] However, inclusion or exclusion of the pair with thoracic MMC did not influence results. Finally, fetal endoscopic treatment is associated with considerable iatrogenic risks, including three fetal deaths.[18] These fetal deaths were caused by severe iatrogenic complications of the fetal endoscopic procedure rather than the result of poor fetal neurological integrity. Although this implies that fetal deaths did not influence our neurological analysis, it should be clearly stated that these risks are to be carefully outweighed before rational clinical treatment choices can be made for future clinical practice.

CONCLUSION

Fetal endoscopic MMC closure could be a promising technique for segmental preservation of neurological leg parameters, but results are achieved at the cost of complications. Before considering clinical implementation of fetal endoscopic MMC closure as standard care, complications should be adequately decreased and results scrutinized in larger study groups over a longer period of time.

Acknowledgements

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CHAPTER 8

Summary and general discussion

Summary

Our main findings presented in the summary concern the influence of the myelomeningocele (MMC) upon the segmental neurologic leg condition caudal to the MMC in spina bifida aperta (SBA). Presented data are obtained by different diagnostic approaches including clinical neurological examination, spinal MRI, leg muscle ultrasound and post-mortem histology. Figure 1 shows the time line at what ages the different investigations in this thesis were performed. Results confirm the existence of two different impacts upon the segmental neurological leg-condition, which are separated in time: 1. 'congenital' damage, evolving as a direct consequence of the neural tube defect and 2. delayed, perinatal damage by secondary spinal trauma. The discussion focuses on the contribution of the muscle ultrasound technique, which may provide an objective, non-invasive quantitative tool for the assessment of neuromuscular damage by the MMC. By age- and lesion matched comparison between different SBA treatment strategies, muscle ultrasound density (MUD) parameters may provide objective quantitative insight into neuromuscular preservation. This information may help the clinician who faces the difficult task to balance the potential risks and benefits associated with different treatment strategies.

In **chapter 1**, we describe the clinical background of neurulation disorders. In addition to cerebral abnormalities, spina bifida aperta is characterized by segmental neurological damage caudal to the MMC. Insight in the underlying pathophysiological mechanisms may provide essential information to choose between innovative, but potentially harmful treatment strategies. Before and after birth, we aimed to elucidate the initiation and evolvement of segmental neuromuscular

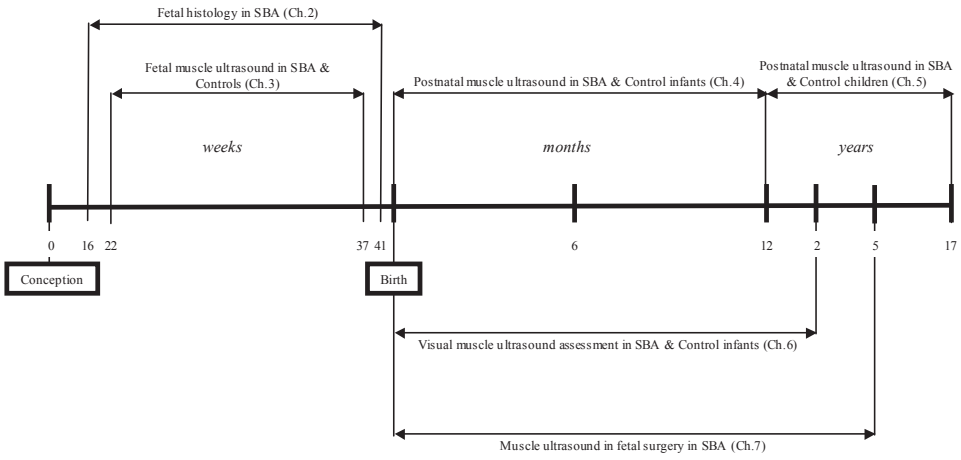


Figure 1. Timeline of histological assessments and muscle ultrasound investigations in spina bifida aperta
The figure provides a schematic overview of the age ranges in which the different investigations were performed. The Roman numerals refer to the corresponding chapters in this thesis.

damage in association with eventual leg motor function loss. To obtain non-invasive insight in leg muscle integrity cranial and caudal to the MMC, we additionally applied the muscle ultrasound technique together with other more conventional diagnostic methods such as clinical neurological examination, spinal radiologic assessment by MRI and post-mortem investigation of the spinal cord and muscles. We aimed to determine whether pre- and postnatal muscle ultrasonography is able to reveal leg muscle abnormalities caudal to the MMC, and, if so, how the initiation and progression of leg muscle ultrasound abnormalities develops over time in relation to leg muscle function loss. Figure 1 shows an overview of the diagnostic evaluations involved in this thesis.

In SBA, fetal leg movements caudal to the MMC are often still present before birth and disappear shortly thereafter. In **chapter 2**, we applied the muscle ultrasound technique to find out whether the early 'congenital' spinal neural tube defect has an impact upon the fetal leg muscle condition caudal to the MMC. We expressed fetal leg muscle ultrasound density (fetal-MUD) as the *ratio* between fetal leg muscle density and bone density. SBA fetuses were shown to have higher MUD outcomes than controls. Furthermore, results indicated that fetal leg-MUD outcomes were independent of gestational age. Combined with the persisting fetal leg movements caudal to the MMC, these non-progressively increased fetal leg-MUD outcomes appeared to reflect 'congenital' leg muscle alterations caused by the MMC.

In **chapter 3**, we described the histological post-mortem findings in succumbed SBA fetuses (16-40 weeks gestational age). We associated perinatal motor function with histological observations of the spinal cord. Despite the congenital neurulation defect, fetal leg movements caudal to the MMC were persistent until the last week of pregnancy. After birth, post-mortem histology revealed evidence for traumatic, delivery-related spinal haemorrhages, superimposed upon the 'congenital' consequences by the neural tube defect. These haemorrhages were located at aberrant spinal blood vessels at, and caudal to the level of the MMC (area vasculorum). Due to the coincidence of these delivery-related spinal segmental haemorrhages (located near persistent lower motor neurons) and early neonatal disappearance of leg movements, a causal relationship appears likely. This delayed, perinatal secondary segmental spinal damage (superimposed upon the congenital neural tube defect) is hypothetically referred to as 'the second-hit of spinal damage'.

In **chapter 4**, we investigated whether lesion-matched postnatal SBA leg-MUD parameters during the first year of life can reveal the evolution of the 'second-hit of spinal damage' and whether outcomes relate with eventual leg muscle function loss. At 0, 6 and 12 months of age, we cross-sectionally compared leg-MUD parameters between SBA and control infants in relation to leg muscle function. To assess the impact of the MMC upon muscle integrity caudal to the MMC, we intra-individually compared the difference in MUD between myotomes caudal- and cranial- to the MMC (dMUD calculated as: [$MUD_{caudal\text{-}to\text{-}the\text{-}MMC}$]-[$MUD_{cranial\text{-}to\text{-}the\text{-}MMC}$]). From the newborn period onwards,

we observed increased SBA leg-dMUD outcomes (reflective of pre-existent, 'congenital' leg muscle alterations). However, at 6 and 12 months of age, we also observed an additional increase in SBA leg-MUD parameters, which finally corresponded with leg muscle dysfunction. During the first year of life, the evolution of SBA leg MUD alterations may thus reflect the impact by both 'congenital' and 'second-hit' spinal damage upon leg muscle integrity. The observed relationship between segmental neurological function and SBA leg-MUD outcomes at 6 and 12 months of age, may thus implicate that SBA leg-MUD could provide an objective, non-invasive, quantitative evaluation tool for post-neonatal assessment of the segmental neurological condition.

In **chapter 5**, we investigated whether postnatal leg-MUD parameters after the first year of life (1-18 years) reflect a 'stabilised' neuromuscular leg condition (i.e. when the ongoing impact by the 'second-hit' of damage is completed). In both SBA and control children, leg-MUD parameters were independent of age. Cross-sectional comparison of leg-MUD parameters revealed higher outcomes in SBA than in control children. Intra-individual SBA leg-MUD parameters differed between both legs. Asymmetrical leg-MUD parameters reflected asymmetrical SBA leg muscle function caudal to the MMC. These data implicate that cross-sectional, lesion-matched leg-MUD parameters are applicable for long-term neurological comparison between different treatment strategies.

In **chapter 6,** we investigated whether the parameter leg-dMUD can also be visually assessed dMUD can be calculated by: dMUD = [$MUD_{calf\text{-}muscle}$]-[$MUD_{quadriceps\text{-}muscle}$]. However, quantitative dMUD assessment requires time, equipment and expertise, whereas it could also be visually determined by differences in muscle echodensity (visual-dMUD). If visual and quantitative dMUD correspond, visual dMUD assessment could provide a quick clinical screening parameter. We therefore investigated whether quantitative dMUD outcomes can also be visually discerned. Results indicated that quantitative dMUD can be visually discerned with an acceptable accuracy (81%) and sensitivity (86%), whereas specificity (57%) appeared relatively low. These characteristics are associated with a 'rule out test', implicating that if a positive quantitative dMUD is present, it will be picked up by visual screening. In SBA children, this may implicate that intra-individual visual dMUD assessment can be performed as a quick and non-invasive screening method to estimate segmental leg muscle damage. However, in case of unexpected findings and/or altered longitudinal visual dMUD outcomes, quantitative confirmation (by the 'golden standard') is necessary.

In **chapter 7**, we investigated whether the neuromuscular impact by the 'second-hit' of spinal damage is reversible by fetal endoscopic neuroprotection. We therefore compared segmental neurological outcomes between age- and lesion matched pairs of SBA children treated by fetal endoscopic and postnatal MMC closure. We reasoned that differences in quantitative dMUD outcomes between age- and lesion matched treatment groups, would reveal whether endoscopic fetal treatment can ameliorate the consequences by the 'second-hit' of damage, or not. Accordingly,

we observed a lower dMUD (reflective of a smaller traumatic MMC impact upon the leg muscle condition) in the fetal endoscopic than in the postnatal treatment group. However, favourable results were obtained at the cost of many perinatal complications, involving prematurity, chorioamnionitis, premature rupture of amniotic membranes, oligohydramnios, infant respiratory distress syndrome necessitating intermittent positive pressure ventilation and even fetal death. From these findings we can conclude that, compared to neonatal surgery, fetal endoscopic MMC surgery is associated with better spinal segmental neuroprotection, but at the cost of iatrogenic damage. Before considering clinical implementation of fetal endoscopic MMC treatment as standard care, the frequency and impact of these complications should be reduced and investigated in larger study groups over a longer period of time.

Key findings

- In SBA fetuses, leg movements caudal to the MMC may persist, but disappear shortly after birth.
- Persistent fetal SBA leg movements concur with non-progressively increased fetal leg-MUD outcomes reflective of a 'congenital' neuromuscular impact by the MMC.
- In SBA, delivery related segmental spinal haemorrhages coincide with early neonatal leg movement loss.
- In SBA infants between 0 and 12 months of age, incremental leg-MUD patterns reflect the impact by both 'congenital' and 'second-hit' spinal damage.
- In SBA children between one and 18 years of age, leg-MUD parameters are age-independent and associated with segmental neurological leg function.
- After fetal endoscopic MMC closure, MUD parameters reveal a more preserved segmental leg muscle condition, but at the cost of a higher complication rate.

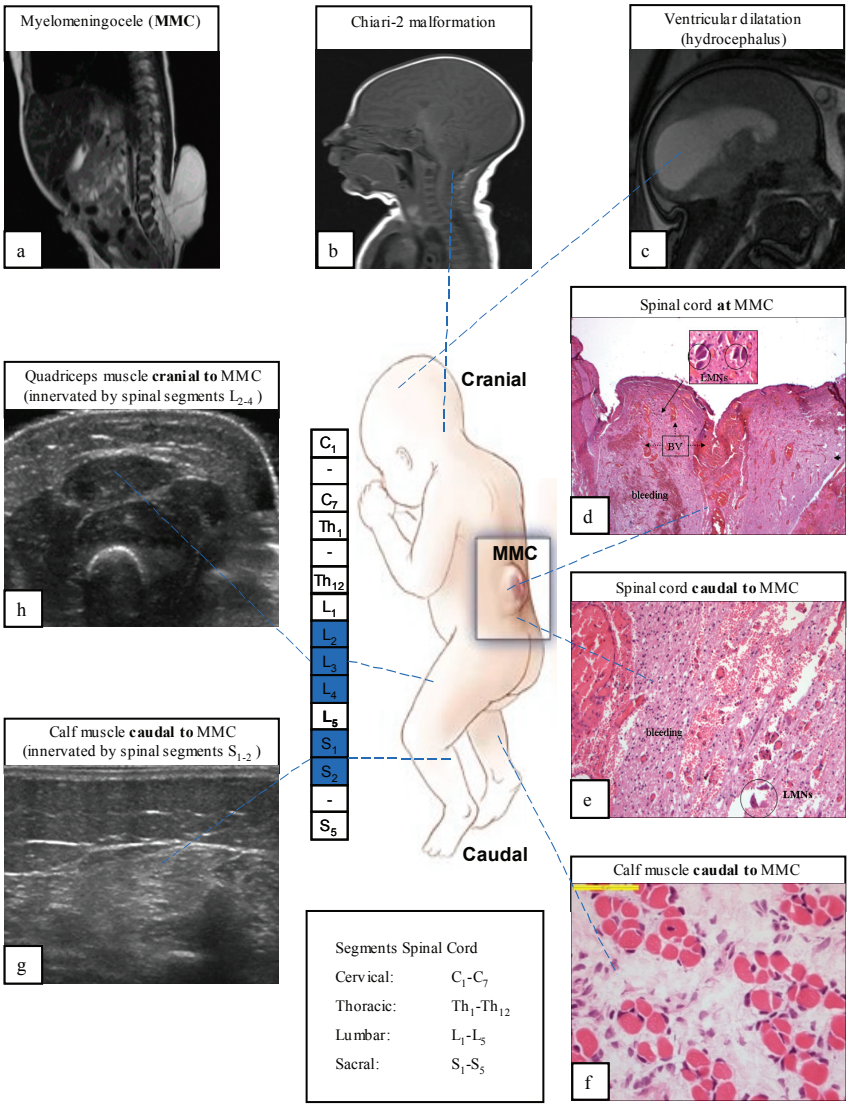
Abbreviations

SBA = spina bifida aperta; MMC = myelomeningocele; MUD = muscle ultrasound density.

GENERAL DISCUSSION

In this thesis, we aimed to elucidate the pathophysiological mechanisms underlying segmental neuromuscular damage caudal to the MMC. Forthcoming data are obtained by different diagnostic approaches and techniques involving segmental neurological examination, spinal assessment by MRI, post-mortem histology and leg muscle ultrasound assessments. A schematic overview of the findings is shown in figure 2. Our results confirm the impact of primary ('congenital') and secondary ('perinatal traumatic') spinal damage. This discussion focuses on the specific diagnostic contribution by the muscle ultrasound technique. Muscle ultrasound may provide an objective, non-invasive

quantitative tool for clinical surveillance and long term comparison between different treatment strategies.



'Second-hit hypothesis'

In SBA, ultrasound studies of perinatal motor behaviour have shown that leg movements are still present before birth, but disappear shortly thereafter.[1,2] This may implicate that the direct cause for leg movement loss becomes manifest perinatally instead of during the early prenatal period. In SBA, these findings are explained by the 'second-hit hypothesis' referring to two different stages of spinal damage.[3-6] The 'first-hit' refers to the impact by the congenital neural tube defect itself

Figure 2. Cerebral, spinal and secondary muscle damage in spina bifida aperta cranial and caudal to the myelomeningocele. Spina bifida aperta (SBA) is congenital neural tube defect characterized by defective fusion of the vertebrae during the first weeks of pregnancy. The incomplete fusion of the vertebral arches is followed by protrusion of neural tissue through the bony defect in a saclike structure. This is called a myelomeningocele (MMC) (a) in which the neural tube defect is accompanied by a congenital malformation of the myelum with protrusion of meninges and spinal cord trough the vertebral defect. In most children, SBA is accompanied by additional cerebral malformations including Chiari-2 malformation (b) and ventricular dilatation (resulting in hydrocephalus (c)). Although the spinal organisation and vascularisation cranial to the MMC is essentially normal in SBA, spinal pathology at (d) and caudal (e) to the level of the MMC involved reduced quantity of lower motor neurons and aberrant spinal blood vessels accompanied by spinal bleedings (d,e). These spinal bleedings (mostly provoked during delivery of the fetus) cause secondary neural damage resulting in muscle alterations caudal to the MMC. Whereas histology of muscles cranial to the MMC was normal, muscles caudal to the MMC revealed histological damage involving decreased water content, muscle fibre atrophy, fat deposition and fibrosis (f). These histological muscle alterations can be assessed by muscle ultrasound in fetuses, infants and children. Muscle damage is associated with an increase in "whiteness" of the muscle which can be quantified by the parameter "muscle ultrasound density" (MUD). For example when a SBA child has a MMC at L_5, the quadriceps muscle (segmental innervation L_{2-4}) is innervated cranial to (above) the spinal level of the MMC and the calf muscle (segmental innervation S_{1-2}) caudal (below) the spinal level of the MMC. The damaged calf muscle innervated caudal to the MMC (g) has a much whiter appearance than the non-damaged quadriceps muscle innervated cranial to the MMC (more black appearance (h)) (*reference image www.ru.nl*).

(i.e. MMC).[3] The 'second-hit' refers to the impact by secondary delayed, perinatal spinal damage, superimposed upon the congenital neural tube defect.[1,4,7] These separate hits of damage are reflected by congenital leg muscle alterations (increased dMUD at birth) and by secondary delayed leg muscle alterations, evolving months after birth (i.e. presumably when structural muscle damage such as atrophy and fibrosis occur).[3,8] Insight in the contribution and timing of the 'second-hit hypothesis' seems important to prevent or ameliorate the neuromuscular impairment.[3,8] Especially the time of initiation and contribution by the secondary delayed, perinatal traumatic spinal damage could provide important information for innovative neuroprotection strategies.

In early postnatally succumbed SBA children, we therefore studied histological leg muscle characteristics caudal to the MMC. Despite persistently present leg movements until birth, histological findings revealed that muscle fibre abnormalities (atrophy and/or compensatory hypertrophy) are already present. These age-independent histological muscle alterations were explained by the impact of 'congenital' spinal damage upon the fetal leg muscle condition.[9,10]

The fetal muscle ultrasound technique provided a first entrance to answer the question whether these histological leg muscle alterations are also associated with non-progressive fetal muscle alterations *in vivo*. From the first trimester of pregnancy onwards, we observed non-progressively increased fetal leg-MUD outcomes during persistently present fetal leg movements.[10] By application of the same muscle ultrasound technique after birth, leg-MUD parameters confirmed the presence of congenitally increased leg-MUD by the congenital spinal defect (reflected by an congenitally increased dMUD compared to controls). In postnatal SBA children (6 months), we additionally observed another increase in MUD parameters *caudal* to the MMC, which coincided with leg muscle

function loss. This delayed increase in MUD caudal to the MMC can be explained by the fact that secondary leg muscle alterations can only be ultrasonically detected when the necessary secondary structural muscle alterations that influence the reflection by the ultrasound beam have developed. Considering the expected time lag between innervational muscle damage and histological muscle alterations it is tempting to speculate that these leg-MUD alterations could be present before six months of age. However, we did not obtain data at earlier time intervals to substantiate this.

The additional damage in SBA children caused by a 'second-hit' was also confirmed by spinal histological post-mortem observations. In the spinal cord of succumbed SBA fetuses and neonates, we identified fresh, delivery related segmental spinal haemorrhages at, and caudal to the MMC. Since such haemorrhages occurred in the vicinity of abnormal, aberrant blood vessels (area vasculorum of the MMC). Hypothetically, we could attribute these haemorrhages to venous stasis by uterine labour contractions and/or direct mechanical compression by vaginal delivery.[4,9] In addition to other prenatal neurotoxic and mechanical traumatic influences, these findings may implicate that delivery-related spinal haemorrhages can contribute to the impact of the 'second-hit' of damage. Interestingly, we also observed spinal haemorrhages at some unexposed, well-covered spinal segments, suggesting that the 'second-hit' damage is not exclusively confined to areas of spinal tissue exposure alone. Beside the presence of irreversible congenital damage and potentially 'inevitable' bleedings, this might explain why fetal coverage of the MMC cannot completely reverse all leg muscle damage.

We therefore conclude that fetal SBA leg movements can persist despite 'congenital' spinal damage. Postnatal leg movement loss is closely related to the occurrence of perinatal delivery-related spinal haemorrhages near clusters of spinal motor neurons.[9]

Neurological significance of SBA leg-MUD parameters

Our muscle ultrasound studies in fetuses, newborns and older children with SBA clearly supported: 1. a pre-existent 'congenital' impact by the spinal neural tube defect (chapter 2),[10] 2. a delayed impact by the 'second-hit' of damage (chapter 3) and 3. stabilisation of leg muscle impairment thereafter (chapter 5).

During these three phases, leg-MUD parameters may provide a useful quantitative tool for the evaluation of the muscle condition caudal to the MMC. However, it should be taken into consideration that the above mentioned data were all cross-sectionally obtained, revealing a considerable inter-individual variation. In individual SBA children, these cross-sectionally obtained leg-MUD outcomes should be interpreted against the background of other neurological and radiological outcome parameters. Before we can conclude whether leg-MUD data can provide individual estimations of motor function, we will have to await the forthcoming results of longitudinal leg-MUD trajectories in SBA and control children.

The inter-individual variation in cross-sectional muscle ultrasound data could be explained by the large variation in SBA lesions both cranial (cerebral and spinal abnormalities) and caudal to the

MMC.[11] Regarding malformations and secondary damage, no SBA child is similar to the other and especially cerebral malformations (Chiari-2 malformations, hydrocephalus) may exert an additional traumatic impact upon both arm and leg movements. This influence of cerebral abnormalities is illustrated by the concurrent presence of abnormal SBA arm movements, which are characterized by poor motor repertoire resulting in impaired fine motor skills and coordination difficulties.[12-14] To isolate the specific traumatic effect by the MMC upon muscle integrity caudal to the MMC, we introduced a new SBA muscle ultrasound parameter, denominated as dMUD. dMUD is defined as the intra-individual difference between MUD caudal and cranial to the MMC (dMUD = [$MUD_{caudal\text{-}to\text{-}the\text{-}MMC}$]-[$MUD_{cranial\text{-}to\text{-}the\text{-}MMC}$]). By introduction of dMUD, we reasoned that additional cerebral influences[12,15] upon the muscle condition caudal to the MMC could be filtered out. In SBA children during the first year of age, we showed that increased dMUD outcomes followed the impact by the 'second-hit' of spinal damage. In age- and lesion matched SBA children receiving different neuroprotective treatment strategies, dMUD could thus provide a useful tool for quantitative comparison of the neuroprotective gain at the MMC. All together, one may conclude that single leg-MUD parameters are reflective of the segmental neurologic leg condition, which is caused by traumatic influences both cranial and caudal to the MMC. Whereas leg-dMUD parameters reflect a part of the damage which is inflicted by the MMC itself. It is important to note that dMUD is influenced by the MMC level. This implicates that cross-sectional dMUD comparison between different treatment groups, can only be done under strict lesion-matched conditions

dMUD may thus provide a useful parameter to compare the spinal neuroprotective effect between different lesion-matched treatment strategies. Quantitative dMUD assessment requires time, equipment and expertise,[16-20] whereas we reasoned that dMUD could also be visually estimated by differences in muscle echo density caudal and cranial to the MMC (visual-dMUD). If visual and quantitative dMUD correspond, visual dMUD assessment could provide a quick and easy clinical screening parameter for neuromuscular damage by the MMC. In this perspective, we compared visual versus quantitative dMUD ('the golden standard') within the same region of interest (ROI). In SBA, we showed that quantitative dMUD outcomes could be visually discerned with an acceptable accuracy (81%) and sensitivity (86%). This sensitivity of visual-dMUD screening appeared higher than the described visual recognition of Heckmatt's scores (71%), involving grade I (normal) to grade IV (high muscle echo intensity with complete loss of bone reflection) muscle abnormalities.[21-23] This may be explained by the simplified task involving discrimination between differences in echogenicity, instead of more complex muscle ultrasound characterisation according to Heckmatt's scales.[22] Although visual dMUD outcomes revealed an acceptable accuracy (81%) and sensitivity (86%), specificity (57%) appeared low. These characteristics are reflective of a 'rule-out test', implicating that if a positive quantitative dMUD is present, it will be picked up by visual screening (in 86% of the cases in our study). Conversely, a negative visual dMUD screening outcome would make a positive quantitative dMUD outcome unlikely (i.e. false negative rate is 13%). In SBA children, these data may implicate that intra-individual visual screening of dMUD could be performed as a quick

and non-invasive screening method for segmental muscle damage by the MMC. However, in case of unexpected findings, altered longitudinal visual dMUD outcomes and/or intended comparison between treatment groups, quantitative evaluation (by the 'golden standard') is still warranted.

Neuroprotective therapies for spina bifida

One of the first neuroprotective strategies that was studied, concerned caesarean section. Several studies have compared neurological outcomes between SBA children born by caesarean section and vaginal delivery.[24-27] The results of one of these studies showed that SBA pregnancies with predefined conditions (i.e. midlumbar cystic MMC) may neurologically benefit from elective caesarean section (before onset of uterus contractions).[27,28] However, other studies did not reveal a beneficial effect by caesarean section upon neurological outcome.[24-26] Since caesarean section in these studies was not electively performed and since included lesions did not apply to midlumbar cystic MMCs, results concerning a potential neuroprotective effect remain unclear.

Consecutive therapies to prevent the 'second-hit' of spinal damage aim at fetal coverage of the MMC.[4,29] In 1994, open human fetal MMC surgery was performed for the first time in the USA, followed by endoscopic fetal approaches leaving the fetus in its own intra-uterine environment.[30] However, this endoscopic approach appeared technically difficult to perform and the American surgeons replaced the endoscopic technique by the open fetal procedure by hysterotomy.[14,30-32] The recent results by the 'Management of Myelomeningocele Study' (MOMS trial 2011) have convincingly shown that open fetal surgery can improve neurological outcome in SBA children.[3,4] In this randomised controlled study, fetally operated SBA children were less shunt dependent (40% versus 82% after postnatal operation) and revealed less extensive hindbrain herniation (Chiari-2 malformation) at 12 months follow-up. Additionally, fetally operated children revealed a significantly better mental and motor function development after 30 months.[33,34] However, on the other hand, open fetal surgery was also associated with an increased iatrogenic risk for both mother and fetus.[33] At the moment (2011), fetal endoscopic MMC closure is performed at the German Center for Fetal Surgery & Minimally Invasive Therapy, University Hospital of Giessen-Marburg, Germany. The aim to restart with fetal endoscopic surgery was to preserve segmental leg function with minimal iatrogenic damage.[35,36] As with open fetal treatment, we showed that fetal endoscopic MMC closure resulted in a partial treatment gain (median motor and sensory gain of two segments), but at the risk of severe iatrogenic complications (such as premature rupture of the amniotic membranes, amnion infection, oligohydramnios, preterm delivery, pulmonary hypoplasia, and fetal death).[37] This implicates that the clinician is facing the challenging task to balance segmental 'benefits' against the considerable complications and even mortality. If future fetal procedures could be improved and if risks for mother and child could be convincingly reduced, fetal endoscopic MMC closure may provide a promising technique for the preservation of neurological integrity.[36]

In the near future, we would especially expect that fetal endoscopic surgery could profit from new developments in biomaterials. Fetal closure of the MMC are performed with the fetus' own dura

and skin or with patches consisting of biomaterials or polytetrafluoroethylene.[33,36,40,41] It is likely that these procedures cause iatrogenic damage, especially when polytetrafluoroethylene patches are used. In this perspective, 'tissue engineering' could provide a less traumatic strategy for fetal MMC coverage. Tissue engineering in spina bifida is a technique in which gelatine hydrogel composites or collagen scaffolds incorporated with growth factors are applied to the MMC defect.[42,43] Until now, it is only experimentally applied in rat and sheep models via hysterotomy. The biomaterials appear to be effective scaffolds resulting in the induction of epidermal ingrowth and cellular adhesion with associated deposition of extracellular matrix.[42,44] Such tissue engineering takes advantage of the regenerative capacity of the fetus with a higher frequency of stem cells in all tissues and higher cell proliferation rates.[45] The use of injectable materials would be even less invasive and could potentially be applied earlier in gestation under ultrasound guidance to maximize neural preservation in SBA.[42] For the improvement of the human fetal SBA techniques, we will have to await further refinements of operation techniques and tissue engineering. Until then, before considering clinical implementation of tissue engineering into fetal endoscopic MMC closure as standard care, we would suggest that forthcoming results should be scrutinized in larger study groups over a longer period of time.

In perspective of endoscopic technical difficulties, the present Dutch focus is still on the open procedure.[3,38] In this perspective, Dutch parents expecting a SBA child may receive standardised counselling for an open fetal MMC closure in Leuven, Belgium (performed by Deprest et al. according to the standard procedure as described by the MOMS trial). To allow objective, rational comparison of risks and benefits between the open and endoscopic fetal surgery approaches, longitudinal comparative studies preferably including the use of objective ultrasound parameters should be awaited.[39]

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Nederlandse samenvatting

De neuraalbuis is de structuur van het embryo waaruit hersenen en ruggenmerg worden gevormd. Indien de vorming en sluiting van de neuraalbuis in de 3e en 4e week van de zwangerschap niet goed verlopen, ontstaat een neuraalbuisdefect. Als dit defect zich op het niveau van het ruggenmerg bevindt, is sprake van een open rug of spina bifida aperta. Bij kinderen met spina bifida aperta is er naast de aanlegstoornis van het ruggenmerg, onvolledige sluiting van één of meerdere ruggenwervels. Het gevolg is een open defect op de rug van het kind waardoor hersenvliezen naar buiten stulpen en een blaas vormen gevuld met zenuwweefsel en hersenvocht. Dit wordt een myelomeningocele genoemd en is de meest voorkomende en ernstigste vorm van spina bifida aperta. Een myelomeningocele gaat gepaard met neurologische uitval onder het niveau van het defect zoals uitval van motoriek en gevoel, alsmede blaas- en darmfunctiestoornissen. De neurologische uitval wordt veroorzaakt door de aanlegstoornis van het ruggenmerg en uittredende wortels met daarnaast verdere schade aan zenuwen en spieren door ontbreken van prikkels uit het ruggenmerg. De ernst van de uitval is afhankelijk van het niveau en de uitgebreidheid van de myelomeningocele. De meeste kinderen met spina bifida aperta hebben ook hersenafwijkingen zoals een Chiari malformatie (uitzakken onderste deel van de kleine hersenen en hersenstam door het achterhoofdsgat) en een waterhoofd (hydrocefalus). Deze hersenafwijkingen kunnen het cognitief functioneren en ook de motoriek van de armen negatief beïnvloeden.

Hoewel spina bifida aperta gepaard gaat met neurologische uitval onder het niveau van de myelomeningocele zien we nog beenbewegingen tijdens de zwangerschap. Deze bewegingen zijn vaak van abnormale kwaliteit, maar kunnen ook normaal zijn. Na de geboorte verdwijnen beenbewegingen geleidelijk, wat het lastig maakt de uiteindelijke motorische functie bij pasgeborenen met spina bifida aperta goed te voorspellen. De verdwijnende beenmotoriek kan uitgelegd worden aan de hand van de 'second-hit hypothese'. Deze hypothese geeft een verklaring hoe de aanlegstoornis van het ruggenmerg (myelomeningocele) leidt tot neurologische uitval. De 'eerste hit' bestaat uit de ontwikkeling van de myelomeningocele waardoor tijdens de zwangerschap al afwijkingen ontstaan aan ruggenmerg, wervels, spieren, zenuwen en huid en blijft het zich ontwikkelende ruggenmerg onbedekt. De 'tweede hit' bestaat uit aanvullende schade door toxische inwerking van vruchtwater en mechanische druk van de baarmoeder op de myelomeningocele.
Om de beenmotoriek te behouden en extra schade aan de myelomeningocele te voorkomen, is in eerdere onderzoeken het effect van het type bevalling op de motoriek onderzocht. Eén studie liet zien dat een keizersnede vóór het inzetten van de weeën een betere neurologische uitkomst geeft dan een vaginale bevalling. In overige studies werd de keizersnede pas na het inzetten van de weeën verricht en werd geen invloed op de neurologische functie gevonden.
Een andere methode om functiestoornissen bij spina bifida aperta mogelijk te beperken is foetale chirurgie. Hierbij zou het afdekken van de myelomeningocele tijdens de zwangerschap secundaire zenuw- en spierschade moeten voorkomen. Onlangs heeft een groot onderzoek in Amerika (MOMS trial; beëindigd in 2011) aangetoond dat foetaal geopereerde spina bifida aperta kinderen een

betere neurologische functie hebben dan kinderen geopereerd na de geboorte. Hoewel afdekking van de myelomeningocele voor de geboorte de impact van secundaire schade kan verminderen biedt het geen 'genezing' van het congenitale defect.

De timing van foetale operaties is van belang om zoveel mogelijk neurologische winst te behalen. Hiervoor is het belangrijk om inzicht te krijgen in het ontstaansmoment en de ontwikkeling van structurele veranderingen in ruggenmerg, zenuwen en spieren van kinderen met spina bifida aperta. Tevens zou dit inzicht kunnen helpen om ouders gerichter informatie te geven betreffende de toekomstige motorische mogelijkheden van hun kind. In dit proefschrift onderzochten we daarom het ontstaan en de progressie van spierschade in relatie tot verlies van motorische functie aan de hand van klinisch neurologisch onderzoek, beeldvormend onderzoek van het ruggenmerg middels MRI, microscopisch weefsel onderzoek bij overleden foetussen en pasgeborenen en spierechografie.

Spierechografie wordt al jaren toegepast als een niet-invasieve methode voor het bepalen van de spierconditie bij kinderen met een spier-/zenuwziekte. Wij veronderstelden dat ook bij kinderen met spina bifida aperta spierveranderingen kunnen worden gemeten. Deze spierveranderingen worden op celniveau gekenmerkt door afname van het watergehalte in de spier, afsterven van spiervezels, en in tweede instantie vervanging daarvan door vet en bindweefsel. Op de spierecho worden deze veranderingen gekwantificeerd door een toename van de parameter spierechodensiteit. Hoe meer schade aan de spier, des te witter is de spier op de echo en des te hoger de densiteit. Een gezonde spier bevat juist veel water, ziet er donker uit op de echo en heeft een lage spierechodensiteit. Op deze manier kan door meting van spierechodensiteit een zieke spier onderscheiden worden van een gezonde spier.

In **hoofdstuk 1** van het proefschrift wordt achtergrondinformatie gegeven over neurale buis defecten en wordt de doelstelling van het onderzoek geformuleerd.

Bij foetussen met spina bifida aperta zijn beenbewegingen onder het niveau van de myelomeningocele nog aanwezig maar deze verdwijnen kort na de geboorte. In **hoofdstuk 2** wordt de spierechotechniek gebruikt om te onderzoeken of de aanlegstoornis van de neurale buis invloed heeft op de foetale spierconditie onder het niveau van de myelomeningocele. De hierbij gebruikte maat voor foetale spierechodensiteit was de verhouding tussen spier- en botdensiteit. Bij spina bifida aperta foetussen onder het niveau van de myelomeningocele vonden we een hogere spierechodensiteit dan in vergelijkbare spieren van controle foetussen. De verhoogde spierechodensiteit onder het niveau van de myelomeningocele was onafhankelijk van de zwangerschapsduur. Deze niet progressief verhoogde densiteit onder het niveau van de MMC in spina bifida aperta foetussen weerspiegelt de 'congenitale' spierveranderingen door de impact van

de myelomeningocele.

In **hoofdstuk 3** beschrijven we de resultaten van microscopisch weefsel (histologisch) onderzoek van spieren, bloedvaten en ruggenmerg van overleden foetussen en pasgeborenen met spina bifida aperta. We vergeleken vooraf met echo-onderzoek vastgestelde foetale beenbewegingen en vroege neonatale motoriek onder het niveau van de myelomeningocele met de histologische bevindingen. Ondanks de myelomeningocele en toxische invloeden van vruchtwater en mechanische druk hierop, bleven beenbewegingen onder het niveau van de myelomeningocele aanwezig tot de laatste week van de zwangerschap. Histologisch onderzoek toonde aanwijzingen voor door de bevalling ontstane bloedingen in het ruggenmerg bovenop al aanwezige 'congenitale' schade door de myelomeningocele. De bloedingen waren gelokaliseerd nabij kwetsbare bloedvaten in ruggenmergsegmenten op en onder het niveau van de myelomeningocele. De locatie van de bloedingen (in de buurt van motorneuronen) maakt een relatie met het verdwijnen van beenmotoriek na de geboorte zeer aannemelijk. Deze secundaire schade door bloedingen in het ruggenmerg (bovenop de aanlegstoornis van het ruggenmerg) staat bekend als de 'second-hit' schade.

In **hoofdstuk 4** onderzochten we of de veranderingen in spierechodensiteit parameters *gedurende het eerste levensjaar* de ontwikkeling van secundaire spierveranderingen weergeeft in het kader van de 'second-hit' hypothese. We vergeleken de echodensiteit van verschillende beenspieren tussen spina bifida en controle kinderen op de leeftijd van 0, 6 en 12 maanden, en keken naar de relatie met uitval van motoriek. Het intra-individuele verschil in densiteit tussen spieren boven en onder het niveau van de myelomeningocele (dMUD = $[MUD_{onder\text{-}MMC}]$-$[MUD_{boven\text{-}MMC}]$), dat schade aan spieren onder dit niveau door de myelomeningocele weergeeft, was op alle leeftijden bij spina bifida kinderen hoger dan bij controles. Dit weerspiegelt de aanwezigheid van 'congenitale' spierveranderingen. Op de leeftijd van 6 en 12 maanden was de spierechodensiteit onder het niveau van de myelomeningocele bij spina bifida aperta kinderen verder toegenomen, wat gepaard ging met motorische uitval van de betreffende spier. Gedurende het eerste levensjaar, weerspiegelen de veranderingen in spierechodensiteit dus de impact van zowel 'congenitale' als 'secundaire' spinale schade op de spierconditie. Gezien de aanwezige relatie tussen neurologische functie en spierechodensiteit op de leeftijd van 6 en 12 maanden, kan het meten van spierechodensiteit een objectief en kwantitatief hulpmiddel zijn voor het bepalen van de segmentale neurologische conditie.

In **hoofdstuk 5** onderzochten we of spierecho parameters in kinderen met spina bifida aperta *na het eerste levensjaar* (1-18 jaar) een gestabiliseerde spierconditie weergeven na de uitwerking van de 'second-hit' schade. In zowel spina bifida als controle kinderen waren spierechodensiteit parameters niet leeftijdsafhankelijk en waren hoger bij spina bifida dan bij controle kinderen. Bij spina bifida

kinderen verschilden spierechodensiteit parameters tussen het meest en minst aangedane been en was een asymmetrische beenspierfunctie geassocieerd met een hogere densiteit in het been met de slechtste spierfunctie. Ook deze data laten zien dat spierechodensiteit parameters gebruikt kunnen worden als indicator voor segmentale neurologische (dys)functie.

In **hoofdstuk 6** onderzochten we of het intra-individuele verschil in densiteit tussen spieren boven en onder het niveau van de myelomeningocele (dMUD) ook visueel bepaald kan worden in plaats van door het te berekenen. Het kwantificeren van spierechodensiteit is een objectieve en sensitieve methode maar vereist ook tijd, software en expertise. Als resultaten van visuele en kwantitatieve dMUD overeenkomen, zou visuele dMUD gebruikt kunnen worden als snelle, klinische screening parameter. We onderzochten hiervoor of kwantitatief berekende dMUD verschillen ook visueel onderscheiden kunnen worden. Resultaten lieten zien dat een kwantitatieve dMUD visueel onderscheiden kan worden met een acceptabele nauwkeurigheid (81%) en sensitiviteit (86%), hoewel de specificiteit (57%) relatief laag was. Deze kenmerken zijn geassocieerd met die van een 'rule out' test, wat betekent dat wanneer een positief kwantitatief verschil in dMUD aanwezig is, dit ook wordt opgemerkt met visuele screening. Voor kinderen met spina bifida aperta kan visuele dMUD beoordeling gebruikt worden als non-invasief screeningsmiddel om een snelle inschatting te maken van de aanwezige segmentale spierschade. In geval van onverwachte bevindingen of veranderingen in longitudinale visuele beoordelingen, moet de kwantitatieve meting als gouden standaard gehandhaafd blijven.

In **hoofdstuk 7** onderzochten we of de schadelijke impact van de 'second-hit' voorkomen kan worden door het voor de geboorte afdekken van de myelomeningocele. Hiervoor vergeleken we neurologische uitkomsten van spina bifida aperta kinderen die een foetale endoscopische operatie hadden ondergaan met die van kinderen bij wie de myelomeningocele na de geboorte was gesloten. We vonden dat de neurologische functie en de hiermee geassocieerde spierconditie beter behouden was bij de foetaal geopereerde kinderen. De keerzijde van foetale chirurgie is dat het gepaard gaat met meer complicaties tijdens zwangerschap en na de geboorte, zoals infecties, vroegtijdig breken van de vliezen, vruchtwatertekort, vroeggeboorte en ademhalingsproblemen bij de pasgeborene. Op basis van deze bevindingen kunnen we concluderen dat foetale endoscopische chirurgie het ruggenmerg weliswaar beter beschermt dan operatieve sluiting na de geboorte maar gepaard gaat met een hoog risico op schade voor zowel moeder als kind als gevolg van de ingreep. Voordat foetale endoscopische chirurgie voor spina bifida ingevoerd kan worden als standaardbehandeling, moeten het aantal en de ernst van de complicaties gereduceerd worden en dienen de behaalde voordelen ook op langere termijn nauwkeurig onderzocht te worden.

In **hoofdstuk 8** worden de bevindingen van onze studies samengevat en besproken. Samengevat ondersteunen de resultaten van het uitgevoerde spierecho- en histologisch onderzoek de

hypothese dat bloedingen in het ruggenmerg vanuit kwetsbare bloedvaten op en onder het niveau van de myelomeningocele tijdens het geboorteproces secundaire schade veroorzaken aan het zenuwweefsel. Als gevolg daarvan treden extra spierveranderingen op bovenop de al aanwezige schade veroorzaakt door de myelomeningocele zelf. Dit resulteert in achteruitgang van motorische functie kort na de geboorte en in het eerste levensjaar. Na het eerste levensjaar lijkt de spierschade zich te stabiliseren. Foetale afdekking van de myelomeningocele kan kwetsbare bloedvaten in ruggenmergsegmenten rond de myelomeningocele beschermen wat leidt tot meer behoud van neurologische functie in vergelijking met kinderen geopereerd na de geboorte. Belangrijk is nu om een zorgvuldige afweging te maken tussen de met de foetale ingreep geassocieerde risico's voor moeder en kind en de mogelijke voordelen voor het kind, alvorens foetale chirurgie te beschouwen als standaardbehandeling voor kinderen bij wie prenataal een spina bifida aperta wordt vastgesteld.

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